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<b>(54) Title:</b> NOVEL OSTEOINDUCTIVE COMPOSITIONS  <b>(57) Abstract</b> <p>Human and bovine bone inductive factor products and processes. The factors may be produced by recombinant techniques and are useful in the research and treatment of bone and periodontal defects.</p>		

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## NOVEL OSTEOINDUCTIVE COMPOSITIONS

The present invention relates to novel proteins and processes for obtaining them. These proteins are capable of inducing cartilage and bone formation.

Background

Bone is a highly specialized tissue characterized by an extensive matrix structure formed of fibrous bundles of the protein collagen, and proteoglycans, noncollagenous proteins, lipids and acidic proteins. The processes of bone formation and renewal/repair of bone tissue, which occur continuously throughout life, are performed by specialized cells. Normal embryonic long bone development is preceded by formation of a cartilage model. Bone growth is presumably mediated by "osteoblasts" (bone-forming cells), while remodeling of bone is apparently accomplished by the joint activities of bone-resorbing cells, called "osteoclasts" and osteoblasts. A variety of osteogenic, cartilage-inducing and bone inducing factors have been described. See, e.g. European patent applications 148,155 and 169,016 for discussions thereof.

Brief Description of the Invention

The present invention provides novel proteins in purified form. Specifically, four of the novel proteins are designated BMP-1, BMP-2 Class I (or BMP-2), BMP-3, and BMP-2 Class II (or BMP-4) wherein BMP is bone morphogenic protein. These proteins are characterized by peptide sequences the same as or substantially homologous to amino acid sequences illustrated in Tables II through VIII below. They are capable of inducing bone formation at a predetermined site. These bone inductive factors are further characterized by biochemical and biological characteristics including activity at a concentration of 10 to 1000ng/gram of bone in an in vivo rat bone formation assay described below. Proteins of this invention may be encoded by the DNA sequences depicted in the Tables or by sequences capable

of hybridizing thereto and coding for polypeptides with bone growth factor biological properties or other variously modified sequences demonstrating such properties.

One of the proteins of the invention is designated BMP-1. A portion of the human BMP-1 or hBMP-1 is characterized by the same or substantially the same peptide sequence as that of amino acid #1 through amino acid #37 of Table V, below which represents a genomic hBMP-1 fragment or amino acid #1 through amino acid #730 of Table VI which represents the hBMP-1 cDNA. hBMP-1 or a related bone inductive factor may be further characterized by at least a portion of these sequences. These peptide sequences are encoded by the same or substantially the same DNA sequence, as depicted in nucleotide #3440 through nucleotide #3550 of Table V and in nucleotide #36 through nucleotide #2225 of Table VI, respectively. These hBMP-1 polypeptides are further characterized by the ability to induce bone formation. hBMP-1 demonstrates activity in an in vivo rat bone formation assay at a concentration of 10 to 1000ng/gram of bone.

The homologous bovine growth factor of the invention, designated bBMP-1, is characterized by a peptide sequence containing the same or substantially the same sequence as that of amino acid #1 through amino acid #37 of Table II below which represents a genomic bBMP-1 fragment. This peptide sequence is encoded by the same or substantially the same DNA sequence as depicted in nucleotide #294 through nucleotide #404 of Table II. The bovine peptide sequence identified in Table II below is also 37 amino acids in length. bBMP-1 is further characterized by the ability to induce bone formation.

Another bone inductive protein composition of the invention is designated BMP-2 Class I (or BMP-2). It is characterized by at least a portion of a peptide sequence the same or substantially the same as that of amino acid #1 through amino acid #396 of Table VII which represents the cDNA hBMP-2 Class I. This peptide sequence is encoded by the same or

substantially the same DNA sequence, as depicted in nucleotide #356 through nucleotide #1543 of Table VII. The human peptide sequence identified in Table VII is 396 amino acids in length. hBMP-2 or related bone inductive proteins may also be characterized by at least a portion of this peptide sequence. hBMP-2 Class I is further characterized by the ability to induce bone formation.

The homologous bovine bone inductive protein of the invention designated bBMP-2 Class I (or bBMP-2), has a DNA sequence identified in Table III below which represents the genomic sequence. This bovine DNA sequence has a prospective 129 amino acid coding sequence followed by approximately 205 nucleotides (a presumptive 3' non-coding sequence). bBMP-2, Class I is further characterized by the ability to induce bone formation. A further bone inductive protein composition of the invention is designated BMP-2 Class II or BMP-4. The human protein hBMP-2 Class II (or hBMP-4) is characterized by at least a portion of the same or substantially the same peptide sequence between amino acid #1 through amino acid #408 of Table VIII, which represents the cDNA of hBMP-2 Class II. This peptide sequence is encoded by at least a portion of the same or substantially the same DNA sequence as depicted in nucleotide #403 through nucleotide #1626 of Table VIII. This factor is further characterized by the ability to induce bone formation.

Still another bone inductive factor of the invention, BMP-3, is represented by the bovine homolog bBMP-3. bBMP-3 is characterized by the DNA sequence and amino acid sequence of Table IV A and B which represents the bovine genomic sequence. It is characterized by at least a portion of a peptide sequence the same or substantially the same as amino acid #1 through amino acid #175 of Table IV A and B. BMP-3 is further characterized by the ability to induce bone formation. The bovine factor may be employed as a tool for obtaining the analogous human BMP-3 protein or other mammalian bone inductive proteins. The proper characterization of this bovine bone



inductive factor provides the essential "starting point" for the method employing this sequence. The method, employing techniques known to those skilled in the art of genetic engineering, involves using the bovine DNA sequence as a probe to screen a human genomic or cDNA library; and identifying the DNA sequences which hybridize to the probes. A clone with a hybridizable sequence is plaque purified and the DNA isolated therefrom, subcloned and subjected to DNA sequence analysis. Thus as another aspect of this invention is a human protein hBMP-3, produced by this method.

Another aspect of the invention provides pharmaceutical compositions containing a therapeutically effective amount of one or more bone growth factor polypeptides according to the invention in a pharmaceutically acceptable vehicle. These compositions may further include other therapeutically useful agents. They may also include an appropriate matrix for delivering the proteins to the site of the bone defect and for providing a structure for bone growth. These compositions may be employed in methods for treating a number of bone defects and periodontal disease. These methods, according to the invention, entail administering to a patient needing such bone formation an effective amount of at least one of the novel proteins BMP-1, BMP-2 Class I, BMP-2 Class-II, and BMP-3 as described herein.

Still a further aspect of the invention are DNA sequences coding on expression for a human or bovine polypeptide having the ability to induce bone formation. Such sequences include the sequence of nucleotides in a 5' to 3' direction illustrated in Tables II through VIII. Alternatively, a DNA sequence which hybridizes under stringent conditions with the DNA sequences of Tables II - VIII or a DNA sequence which hybridizes under non-stringent conditions with the illustrated DNA sequences and which codes on expression for a protein having at least one bone growth factor biological property are included in the present invention. Finally, allelic or other variations of the

sequences of Tables II through VIII, whether such nucleotide changes result in changes in the peptide sequence or not, are also included in the present invention.

Still a further aspect of the invention is a vector containing a DNA sequence as described above in operative association with an expression control sequence. Such vector may be employed in a novel process for producing a bone growth factor polypeptide in which a cell line transformed with a DNA sequence encoding expression of a bone growth factor polypeptide in operative association with an expression control sequence therefor, is cultured. This claimed process may employ a number of known cells as host cells for expression of the polypeptide. Presently preferred cell lines are mammalian cell lines and bacterial cells.

Other aspects and advantages of the present invention will be apparent upon consideration of the following detailed description and preferred embodiments thereof.

#### Detailed Description of the Invention

The proteins of the present invention are characterized by amino acid sequences or portions thereof the same as or substantially homologous to the sequences shown in Tables II - VIII below. These proteins are also characterized by the ability to induce bone formation.

The bone growth factors provided herein also include factors encoded by the sequences similar to those of Tables II - VIII, but into which modifications are naturally provided (e.g. allelic variations in the nucleotide sequence which may result in amino acid changes in the polypeptide) or deliberately engineered. For example, synthetic polypeptides may wholly or partially duplicate continuous sequences of the amino acid residues of Tables II - VIII. These sequences, by virtue of sharing primary, secondary, or tertiary structural and conformational characteristics with bone growth factor polypeptides of Tables II - VIII may possess bone growth factor biological properties in common therewith. Thus, they may be

employed as biologically active substitutes for naturally-occurring bone growth factor polypeptides in therapeutic processes.

Other specific mutations of the sequences of the bone growth factors described herein involve modifications of one or both of the glycosylation sites. The absence of glycosylation or only partial glycosylation results from amino acid substitution or deletion at one or both of the asparagine-linked glycosylation recognition sites present in the sequences of the bone growth factors shown in Tables II-VIII.

The asparagine-linked glycosylation recognition sites comprise tripeptide sequences which are specifically recognized by appropriate cellular glycosylation enzymes. These tripeptide sequences are either asparagine-X-threonine or asparagine-X-serine, where X is usually any amino acid. A variety of amino acid substitutions or deletions at one or both of the first or third amino acid positions of a glycosylation recognition site (and/or amino acid deletion at the second position) results in non-glycosylation at the modified tripeptide sequence.

The present invention also encompasses the novel DNA sequences, free of association with DNA sequences encoding other proteinaceous materials, and coding on expression for bone growth factors. These DNA sequences include those depicted in Tables II - VIII in a 5' to 3' direction and those sequences which hybridize under stringent hybridization conditions [see, T. Maniatis et al, Molecular Cloning (A Laboratory Manual), Cold Spring Harbor Laboratory (1982), pages 387 to 389] to the DNA sequences of Tables II - VIII.

DNA sequences which hybridize to the sequences of Tables II - VIII under relaxed hybridization conditions and which code on expression for bone growth factors having bone growth factor biological properties also encode bone growth factors of the invention. For example, a DNA sequence which shares regions of significant homology, e.g., sites of glycosylation



or disulfide linkages, with the sequences of Tables II - VIII and encodes a bone growth factor having one or more bone growth factor biological properties clearly encodes a member of this novel family of growth factors, even if such a DNA sequence would not stringently hybridize to the sequence of Tables II - VIII.

Similarly, DNA sequences which code for bone growth factor polypeptides coded for by the sequences of Tables II - VIII, but which differ in codon sequence due to the degeneracies of the genetic code or allelic variations (naturally-occurring base changes in the species population which may or may not result in an amino acid change) also encode the novel growth factors described herein. Variations in the DNA sequences of Tables II - VIII which are caused by point mutations or by induced modifications to enhance the activity, half-life or production of the polypeptides encoded thereby are also encompassed in the invention.

Another aspect of the present invention provides a novel method for producing the novel osteoinductive factors. The method of the present invention involves culturing a suitable cell or cell line, which has been transformed with a DNA sequence coding on expression for a novel bone growth factor polypeptide of the invention, under the control of known regulatory sequences. Suitable cells or cell lines may be mammalian cells, such as Chinese hamster ovary cells (CHO). The selection of suitable mammalian host cells and methods for transformation, culture, amplification, screening and product production and purification are known in the art. See, e.g., Gething and Sambrook, Nature, 293:620-625 (1981), or alternatively, Kaufman et al, Mol. Cell. Biol., 5(7):1750-1759 (1985) or Howley et al, U.S. Patent 4,419,446. Another suitable mammalian cell line, which is described in the accompanying examples, is the monkey COS-1 cell line. A similarly useful mammalian cell line is the CV-1 cell line.

Bacterial cells are suitable hosts. For example, the

various strains of E. coli (e.g., HB101, MC1061) are well-known as host cells in the field of biotechnology. Various strains of B. subtilis, Pseudomonas, other bacilli and the like may also be employed in this method.

Many strains of yeast cells known to those skilled in the art are also available as host cells for expression of the polypeptides of the present invention. Additionally, where desired, insect cells may be utilized as host cells in the method of the present invention. See, e.g. Miller et al, Genetic Engineering, 8:277-298 (Plenum Press 1986) and references cited therein.

Another aspect of the present invention provides vectors for use in the method of expression of these novel osteoinductive polypeptides. Preferably the vectors contain the full novel DNA sequences described above which code for the novel factors of the invention. Additionally the vectors also contain appropriate expression control sequences permitting expression of the bone-inductive protein sequences. Alternatively, vectors incorporating modified sequences as described above are also embodiments of the present invention and useful in the production of the bone inductive proteins. The vectors may be employed in the method of transforming cell lines and contain selected regulatory sequences in operative association with the DNA coding sequences of the invention which are capable of directing the replication and expression thereof in selected host cells. Useful regulatory sequences for such vectors are known to one of skill in the art and may be selected depending upon the selected host cells. Such selection is routine and does not form part of the present invention.

A protein of the present invention, which induces bone growth in circumstances where bone is not normally formed, has application in the healing of bone fractures. An osteogenic preparation employing one or more of the proteins of the invention may have prophylactic use in closed as well as open

fracture reduction and also in the improved fixation of artificial joints. De novo bone formation induced by an osteogenic agent contributes to the repair of congenital, trauma induced, or oncologic resection induced craniofacial defects, and also is useful in cosmetic plastic surgery. An osteogenic factor of the invention may be valuable in the treatment of periodontal disease, and in other tooth repair processes. Such agents may provide an environment to attract bone-forming cells, stimulate growth of bone-forming cells or induce differentiation of progenitors of bone-forming cells. Of course, the proteins of the invention may have other therapeutic uses.

A further aspect of the invention is a therapeutic method and composition for repairing fractures and other conditions related to bone defects or periodontal diseases. Such a composition comprises a therapeutically effective amount of at least one of the bone inductive factor proteins of the invention. —The bone inductive factors according to the present invention may be present in a therapeutic composition in admixture with a pharmaceutically acceptable vehicle or matrix. Further therapeutic methods and compositions of the invention comprise a therapeutic amount of a bone inductive factor of the invention with a therapeutic amount of at least one of the other bone inductive factors of the invention. Additionally, the proteins according to the present invention or a combination of the proteins of the present invention may be co-administered with one or more different osteoinductive factors with which it may interact. Further, the bone inductive proteins may be combined with other agents beneficial to the treatment of the bone defect in question. Such agents include, but are not limited to various growth factors. The preparation of such physiologically acceptable protein compositions, having due regard to pH, isotonicity, stability and the like, is within the skill of the art.

In particular, BMP-1 may be used individually in a

composition. BMP-1 may also be used in combination with one or more of the other proteins of the invention. BMP-1 and BMP-2 Class I may be used in combination. BMP-1 and BMP-2 Class II may also be used in combination. BMP-1 and BMP-3 may be used in combination. Furthermore, BMP-1 may be used in combination with two or three of the other proteins of the invention. For example, BMP-1, BMP-2 Class I, and BMP-2 Class II may be combined. BMP-1 may also be combined with BMP-2 Class I, and BMP-3. Further, BMP-1 may be combined with BMP-2 Class II, and BMP-3. BMP-1, BMP-2 Class I, BMP-2 Class II, and BMP-3 may be combined.

BMP-2 Class I may be used individually in a pharmaceutical composition. BMP-2 Class I may also be used in combination with one or more of the other proteins of the invention. BMP-2 Class I may be combined with BMP-2 Class II. It may also be combined with BMP-3. Further BMP-2 Class I may be combined with BMP-2 Class II and BMP-3.

----- BMP-2 Class II may be used individually in pharmaceutical composition. In addition, it may be used in combination with other proteins as identified above. Further it may be used in combination with BMP-3.

BMP-3 may be used individually in a composition. It may further be used in the various combinations identified above.

The therapeutic method includes locally administering the composition as an implant or device. When administered, the therapeutic composition for use in this invention is, of course, in a pyrogen-free, physiologically acceptable form. Further, the composition may desirably be encapsulated or injected in a viscous form for delivery to the site of bone damage. Preferably, the bone growth inductive factor composition would include a matrix capable of delivering the bone inductive factor to the site of bone damage, providing a structure for the developing bone and cartilage and optimally capable of being resorbed into the body. Such matrices may be formed of other materials presently in use for other implanted medical



applications.

The choice of material is based on, for example, biocompatibility, biodegradability, mechanical properties, cosmetic appearance and interface properties. Similarly, the application of the osteoinductive factors will define the appropriate formulation. Potential matrices for the osteoinductive factors may be biodegradable and chemically defined, such as, but not limited to calcium sulfate, tricalciumphosphate, hydroxyapatite, polylactic acid, polyanhydrides; biodegradable and biologically well defined, such as bone or dermal collagen, other pure proteins or extracellular matrix components; nonbiodegradable and chemically defined, such as sintered hydroxyapatite, bioglass, aluminates, or other ceramics; or combinations of any of the above mentioned types of material, such as polylactic acid and hydroxyapatite or collagen and tricalciumphosphate. The bioceramics might also be altered in composition, such as in calcium-aluminate-phosphate and processing to alter for example, pore size, particle size, particle shape, and biodegradability.

The dosage regimen will be determined by the attending physician considering various factors which modify the action of such a growth factor, e.g. amount of bone weight desired to be formed, the site of bone damage, the condition of the damaged bone, the patient's age, sex, and diet, the severity of any infection, time of administration and other clinical factors. The dosage may vary with the type of matrix used in the reconstitution and the composition of BMP's. The addition of other known growth factors, such as IGF 1 (insulin like growth factor 1), to the final composition, may also effect the dosage. Generally, the dosage regimen should be in the range of approximately  $10$  to  $10^6$  nanograms of protein per gram of bone weight desired. Progress can be monitored by periodic assessment of bone growth and/or repair, e.g. x-rays. Such therapeutic compositions are also presently valuable for veterinary applications due to the lack of species specificity



in bone inductive factors. Particularly domestic animals and thoroughbred horses in addition to humans are desired patients for such treatment with the bone inductive factors of the present invention.

The following examples illustrate practice of the present invention in recovering and characterizing the bovine proteins and employing them to recover the human proteins, obtaining the human proteins and in expressing the proteins via recombinant techniques.

### EXAMPLE I

#### Isolation of Bovine Bone Inductive Factor

Ground bovine bone powder (20-120 mesh, Helitrex) is prepared according to the procedures of M. R. Urist et al., Proc. Natl Acad. Sci USA, 70:3511 (1973) with elimination of some extraction steps as identified below. Ten kgs of the ground powder is demineralized in successive changes of 0.6N HCl at 4°C over a 48-hour period with vigorous stirring. The resulting suspension is extracted for 16 hours at 4°C with 50 liters of 2M CaCl<sub>2</sub> and 10mM ethylenediamine-tetraacetic acid [EDTA], and followed by extraction for 4 hours in 50 liters of 0.5M EDTA. The residue is washed three times with distilled water before its resuspension in 20 liters of 4M guanidine hydrochloride [GuCl], 20mM Tris (pH 7.4), 1mM N-ethylmaleimide, 1mM iodoacetamide, 1mM phenylmethylsulfonyl fluoride as described in Clin. Orthop. Rel. Res., 171: 213 (1982). After 16 to 20 hours the supernatant is removed and replaced with another 10 liters of GuCl buffer. The residue is extracted for another 24 hours.

The crude GuCl extracts are combined, concentrated approximately 20 times on a Pellicon apparatus with a 10,000 molecular weight cut-off membrane, and then dialyzed in 50mM Tris, 0.1M NaCl, 6M urea (pH7.2), the starting buffer for the first column. After extensive dialysis the protein is loaded on a 4 liter DEAE cellulose column and the unbound fractions

are collected.

The unbound fractions are concentrated and dialyzed against 50mM NaAc, 50mM NaCl (pH 4.6) in 6M urea. The unbound fractions are applied to a carboxymethyl cellulose column. Protein not bound to the column is removed by extensive washing with starting buffer, and the bone inductive factor containing material desorbed from the column by 50mM NaAc, 0.25mM NaCl, 6M urea (pH 4.6). The protein from this step elution is concentrated 20- to 40- fold, then diluted 5 times with 80mM KPO<sub>4</sub>, 6M urea (pH6.0). The pH of the solution is adjusted to 6.0 with 500mM K<sub>2</sub>HPO<sub>4</sub>. The sample is applied to an hydroxylapatite column (LKB) equilibrated in 80mM KPO<sub>4</sub>, 6M urea (pH6.0) and all unbound protein is removed by washing the column with the same buffer. Bone inductive factor activity is eluted with 100mM KPO<sub>4</sub> (pH7.4) and 6M urea.

The protein is concentrated approximately 10 times, and solid NaCl added to a final concentration of 0.15M. This material is applied to a heparin - Sepharose column equilibrated in 50mM KPO<sub>4</sub>, 150mM NaCl, 6M urea (pH7.4). After extensive washing of the column with starting buffer, a protein with bone inductive factor activity is eluted by 50mM KPO<sub>4</sub>, 700mM NaCl, 6M urea (pH7.4). This fraction is concentrated to a minimum volume, and 0.4ml aliquots are applied to Superose 6 and Superose 12 columns connected in series, equilibrated with 4M GuCl, 20mM Tris (pH7.2) and the columns developed at a flow rate of 0.25ml/min. The protein demonstrating bone inductive factor activity has a relative migration corresponding to approximately 30,000 dalton protein.

The above fractions are pooled, dialyzed against 50mM NaAc, 6M urea (pH4.6), and applied to a Pharmacia MonoS HR column. The column is developed with a gradient to 1.0M NaCl, 50mM NaAc, 6M urea (pH4.6). Active fractions are pooled and brought to pH3.0 with 10% trifluoroacetic acid (TFA). The material is applied to a 0.46 x 25cm Vydac C4 column in 0.1% TFA and the column developed with a gradient to 90% acetonitrile, 0.1% TFA

(31.5% acetonitrile, 0.1% TFA to 49.5% acetonitrile, 0.1% TFA in 60 minutes at 1ml per minute). Active material is eluted at approximately 40-44% acetonitrile. Aliquots of the appropriate fractions are iodinated by one of the following methods: P. J. McConahey et al, Int. Arch. Allergy, 29:185-189 (1966); A. E. Bolton et al, Biochem J., 133:529 (1973); and D. F. Bowen-Pope, J. Biol. Chem., 237:5161 (1982). The iodinated proteins present in these fractions are analyzed by SDS gel electrophoresis and urea Triton X 100 isoelectric focusing. At this stage, the bone inductive factor is estimated to be approximately 10-50% pure.

## EXAMPLE II

### Characterization of Bovine Bone Inductive Factor

#### A. Molecular Weight

Approximately 20ug protein from Example I is lyophilized and redissolved in 1X SDS sample buffer. After 15 minutes of heating at 37°C, the sample is applied to a 15% SDS polyacrylamide gel and then electrophoresed with cooling. The molecular weight is determined relative to prestained molecular weight standards (Bethesda Research Labs). Immediately after completion, the gel lane containing bone inductive factor is sliced into 0.3cm pieces. Each piece is mashed and 1.4ml of 0.1% SDS is added. The samples are shaken gently overnight at room temperature to elute the protein. Each gel slice is desalted to prevent interference in the biological assay. The supernatant from each sample is acidified to pH 3.0 with 10% TFA, filtered through a 0.45 micron membrane and loaded on a 0.46cm x 5cm C4 Vydac column developed with a gradient of 0.1% TFA to 0.1% TFA, 90% CH<sub>3</sub>CN. The appropriate bone inductive factor - containing fractions are pooled and reconstituted with 20mg rat matrix. In this gel system, the majority of bone inductive factor fractions have the mobility of a protein having a molecular weight of approximately 28,000 - 30,000 daltons.

### B. Isoelectric Focusing

The isoelectric point of bone inductive factor activity is determined in a denaturing isoelectric focusing system. The Triton X100 urea gel system (Hoeffer Scientific) is modified as follows: 1) 40% of the ampholytes used are Servalyte 3/10; 60% are Servalyte 7-9. 2) The catholyte used is 40mM NaOH. Approximately 20ug of protein from Example I is lyophilized, dissolved in sample buffer and applied to the isoelectrofocusing gel. The gel is run at 20 watts, 10°C for approximately 3 hours. At completion the lane containing bone inductive factor is sliced into 0.5 cm slices. Each piece is mashed in 1.0ml 6M urea, 5mM Tris (pH 7.8) and the samples agitated at room temperature. The samples are acidified, filtered, desalted and assayed as described above. The major portion of activity as determined in the assay described in Example III migrates in a manner consistent with a pI of 8.8 - 9.2.

### C. Subunit Characterization

The subunit composition of bone inductive factor is also determined. Pure bone inductive factor is isolated from a preparative 15% SDS gel as described above. A portion of the sample is then reduced with 5mM DTT in sample buffer and re-electrophoresed on a 15% SDS gel. The approximately 30kd protein yields two major bands at approximately 20kd and 18kd, as well as a minor band at 30kd. The broadness of the two bands indicates heterogeneity caused most probably by glycosylation, other post translational modification, proteolytic degradation or carbamylation.

## EXAMPLE III

### Biological Activity of Bone Inductive Factor

A rat bone formation assay according to the general procedure of Sampath and Reddi, Proc. Natl. Acad. Sci. U.S.A.,

80:6591-6595 (1983) is used to evaluate the osteogenic activity of the bovine bone inductive factor of the present invention obtained in Example I. This assay can also be used to evaluate bone inductive factors of other species. The ethanol precipitation step is replaced by dialyzing the fraction to be assayed against water. The solution or suspension is then redissolved in a volatile solvent, e.g. 0.1 - 0.2 % TFA, and the resulting solution added to 20mg of rat matrix. This material is frozen and lyophilized and the resulting powder enclosed in #5 gelatin capsules. The capsules are implanted subcutaneously in the abdominal thoracic area of 21 - 49 day old male long Evans rats. The implants are removed after 7 - 14 days. Half of each implant is used for alkaline phosphatase analysis [See, A. H. Reddi et al., Proc. Natl Acad Sci., 69:1601 (1972)] and half is fixed and processed for histological analysis. Routinely, 1um glycolmethacrylate sections are stained with Von Kossa and acid fuschin to detect new bone mineral. Alkaline phosphatase, an enzyme produced by chondroblasts and osteoblasts in the process of matrix formation, is also measured. New cartilage and bone formation often correlates with alkaline phosphatase levels. Table I below illustrates the dose response of the rat matrix samples including a control not treated with bone inductive factor.

TABLE 1

<u>Protein*</u> <u>Implanted ug</u>	<u>Cartilage</u>	<u>Alk. Phos.u/l</u>
7.5	2	Not done
2.5	3	445.7
0.83	3	77.4
0.28	0	32.5
0.00	0	31.0

\*At this stage the bone inductive factor is approximately 10-15% pure.

The bone or cartilage formed is physically confined to the space occupied by the matrix. Samples are also analyzed by SDS gel electrophoresis and isoelectric focusing as described



above, followed by autoradiography. Analysis reveals a correlation of activity with protein bands at 28 - 30kd and a pI 9.0. An extinction coefficient of 1 OD/mg-cm is used as an estimate for protein and approximating the purity of bone inductive factor in a particular fraction. In the in vivo rat bone formation assays on dilutions as described above, the protein is active in vivo at 10 to 200ng protein/gram bone to probably greater than 1ug protein/gram bone.

#### EXAMPLE IV

##### Bovine Bone Inductive Factor Protein Composition

The protein composition of Example IIA of molecular weight 28 - 30kd is reduced as described in Example IIC and digested with trypsin. Eight tryptic fragments are isolated by standard procedures having the following amino acid sequences:

Fragment 1: A A F L G D I A L D E E D L G

Fragment 2: A F Q V Q Q A A D L

Fragment 3: N Y Q D M V V E G

Fragment 4: S T P A Q D V S R

Fragment 5: N Q E A L R

Fragment 6: L S E P D P S H T L E E

Fragment 7: F D A Y Y

Fragment 8: L K P S N ? A T I Q S I V E

A less highly purified preparation of protein from bovine bone is prepared according to a purification scheme similar to that described in Example I. The purification basically varies from that previously described by omission of the DE-52 column, the CM cellulose column and the mono S column, as well as a reversal in the order of the hydroxylapatite and heparin sepharose columns. Briefly, the concentrated crude 4 M extract is brought to 85% final concentration of ethanol at 4 degrees. The mixture is then centrifuged, and the precipitate redissolved in 50 mM Tris, 0.15 M NaCl, 6.0 M urea. This material is then fractionated on Heparin Sepharose as described. The Heparin bound material

is fractionated on hydroxyapatite as described. The active fractions are pooled, concentrated, and fractionated on a high resolution gel filtration (TSK 30000 in 6 M guanidinium chloride, 50 mM Tris, pH 7.2). The active fractions are pooled, dialyzed against 0.1% TFA, and then fractionated on a C4 Vydac reverse phase column as described. The preparation is reduced and electrophoresed on an acrylamide gel. The protein corresponding to the 18K band is eluted and digested with trypsin. Tryptic fragments are isolated having the following amino acid sequences:

Fragment 9: S L K P S N H A T I Q S ? V

Fragment 10: S F D A Y Y C S ? A

Fragment 11: V Y P N M T V E S C A

Fragment 12: V D F A D I ? W

Tryptic Fragments 7 and 8 are noted to be substantially the same as Fragments 10 and 9, respectively.

#### A. bBMP-1

Probes consisting of pools of oligonucleotides (or unique oligonucleotides) are designed according to the method of R. Lathe, J. Mol. Biol., 183 (1):1-12 (1985) and synthesized on an automated DNA synthesizer. One probe consists of a relatively long (32 nucleotides) "guessmer" [See J. J. Toole et al, Nature, 312:342-347 (1984)] of the following nucleotide sequence:

TCCTCATCCAGGGCAATGTCGCCCAGGAAGGC

Because the genetic code is degenerate (more than one codon can code for the same amino acid), the number of oligonucleotides in a probe pool is reduced based on the frequency of codon usage in eukaryotes, the relative stability of G:T base pairs, and the relative infrequency of the dinucleotide CpG in eukaryotic coding sequences [see Toole et al., *supra.*]. The second set of probes consists of shorter oligonucleotides (17 nucleotides in length) which contain all possible sequences that could encode the amino acids. The second set of probes has the following sequences:

- (a) A [A/G] [A/G] TC [T/C] TC [T/C] TC [A/G] TC [T/C] AA  
 (b) A [A/G] [A/G] TC [T/C] TC [T/C] TC [A/G] TCNAG

Bracketed nucleotides are alternatives. "N" means either A, T, C or G.

In both cases the regions of the amino acid sequence used for probe design are chosen by avoiding highly degenerate codons where possible. The oligonucleotides are synthesized on an automated DNA synthesizer; the probes are then radioactively labeled with polynucleotide kinase and  $^{32}\text{P}$ -ATP.

These two sets of probes are used to screen a bovine genomic recombinant library. The library is constructed as follows: Bovine liver DNA is partially digested with the restriction endonuclease enzyme Sau 3A and sedimented through a sucrose gradient. Size fractionated DNA in the range of 15-30kb is then ligated to the bacteriophage Bam HI vector EMBL3 [Frischauf et al, J. Mol. Biol., 170:827-842 (1983)]. The library is plated at 8000 recombinants per plate. Duplicate nitrocellulose replicas of the plaques are made and amplified according to a modification of the procedure of Woo et al, Proc. Natl. Acad. Sci. USA, 75:3688-91 (1978).

The 32 mer probe is kinased with  $^{32}\text{P}$ -gamma-ATP and hybridized to one set of filters in 5X SSC, 0.1% SDS, 5X Denhardts, 100ug/ml salmon sperm DNA at 45 degrees C and washed with 5X SSC, 0.1% SDS at 45 degrees C. The 17 mer probes are kinased and hybridized to the other set of filters in 3M tetramethylammonium chloride (TMAC), 0.1M sodium phosphate pH6.5, 1mM EDTA, 5X Denhardts, 0.6% SDS, 100ug/ml salmon sperm DNA at 48 degrees C, and washed in 3M TMAC, 50mM Tris pH8.0 at 50 degrees C. These conditions minimize the detection of mismatches to the 17 mer probe pool [see, Wood et al, Proc. Natl. Acad. Sci. U.S.A., 82:1585-1588 (1985)]. 400,000 recombinants are screened by this procedure and one duplicate positive is plaque purified. DNA is isolated from a plate lysate of this recombinant bacteriophage designated lambda bP-50. bP-50 was deposited December 16, 1986 with the American

Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland USA (hereinafter the "ATCC") under accession number 40295. This deposit as well as the other deposits contained herein meets the requirements of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and Regulations thereunder. This bp-50 clone encodes at least a portion of the bovine bone growth factor designated bBMP-1.

The oligonucleotide hybridizing region of this bBMP-1 clone is localized to an approximately 800bp Eco RI fragment which is subcloned into M13 and sequenced by standard techniques. The partial DNA sequence and derived amino acid sequence of lambda bP-50 are shown below in Table II. The amino acid sequences corresponding to the tryptic fragments isolated from the bovine bone 28 to 30kd material are underlined in Table II. The first underlined portion of the sequence corresponds to tryptic Fragment 1 above from which the oligonucleotide probes are designed. The second underlined portion corresponds to tryptic Fragment 2 above. The predicted amino acid sequence indicates that tryptic Fragment 2 is preceded by a basic residue (R) as expected considering the specificity of trypsin. The nucleic acid sequence preceding the couplet CT at nucleotide positions #292-293 in Table II is presumed to be an intron (noncoding sequence) based on the presence of a consensus acceptor sequence (i.e., a pyrimidine rich tract, TCTCTCTCC, followed by AG) and the lack of a basic residue in the appropriate position of the derived amino acid sequence. This bBMP-1 genomic sequence appears in Table II. The presumptive bBMP-1 peptide sequence from this genomic clone is 37 amino acids in length and is encoded by the DNA sequence from nucleotide #294 through #404 in Table II.

TABLE II

280	290	(1)	308	323
CCTTGCCCTCT	TCTCTCTCCA	GCT	GCC TTC CTT GGG GAC ATC GGC CTG GAC GAG GAG	
			<u>Ala Phe Leu Gly Asp Ile Ala Leu Asp Glu Glu</u>	
	338		353	368
GAC TTG AGG GGC TTC CAA GTG CAG CAG GCT GCG GAC CTC AGA CAG CGT GCA ACC				
<u>Asp Leu Arg Ala Phe Gln Val Gln Gln Ala Ala Asp Leu Arg Gln Arg Ala Thr</u>				
383:	398	(37)	414	424
CGC AGG TCT TCC ATC AAA GCT GCA GGTACACTGG GTACAGGCCA				
Arg Arg Ser Ser Ile Lys Ala Ala				



**B. bBMP-2**

Two probes consisting of pools of oligonucleotides are designed on the basis of the amino acid sequence of Fragment 3 and synthesized on an automated DNA synthesizer as described above.

Probe #1: A C N A C C A T [A/G] T C [T/C] T G [A/G] A T

Probe #2: C A [A/G] G A [T/C] A T G G T N G T N G A

These probes are radioactively labeled and employed to screen the bovine genomic library constructed as described in part A except that the vector is lambda J1 Bam HI arms [Mullins et al Nature 308: 856-858 (1984).] The radioactively labelled 17-mer Probe #1 is hybridized to the set of filters according to the method for the 17 mer probe described in part A.

400,000 recombinants are screened by the procedure described above in Part A. One duplicate positive is plaque purified and the DNA is isolated from a plate lysate of the recombinant bacteriophage designated lambda bP-21. Bacteriophage bP-21 was deposited with the ATCC under accession number ATCC 40310 on March 6, 1987. The bP-21 clone encodes the bovine growth factor designated bBMP-2.

The oligonucleotide hybridizing region of this bBMP-2 clone is localized to an approximately 1.2 kb Sac I restriction fragment which is subcloned into M13 and sequenced by standard techniques. The partial DNA sequence and derived amino acid sequence of this Sac I fragment and the contiguous Hind III-

Sac I restriction fragment of bP-21 are shown below in Table III. The bBMP-2 peptide sequence from this clone is 129 amino acids in length and is encoded by the DNA sequence from nucleotide #1 through nucleotide #387. The amino acid sequence corresponding to the tryptic fragment isolated from the bovine bone 28 to 30kd material is underlined in Table III. The underlined portion of the sequence corresponds to tryptic Fragment 3 above from which the oligonucleotide probes for bBMP-2 are designed. The predicted amino acid sequence indicates that tryptic Fragment 3 is preceded by a basic

residue (K) as expected considering the specificity of trypsin. The arginine residue encoded by the CGT triplet is presumed to be the carboxy-terminus of the protein based on the presence of a stop codon (TAG) adjacent to it.

TABLE III

(1)	15	30	45	
GGC CAC GAT GGG AAA GGA CAC CCT CTC CAC AGA AGA GAA AAG CGG				
G H D G K G H P L H R R E K R				
60	75	90		
CAA GCA AAA CAC AAA CAG CGG AAA CGC CTC AAG TCC AGC TGT AAG				
Q A K H K Q R K R L K S S C K				
105	120	135		
AGA CAC CCT TTA TAT GTG GAC TTC AGT GAT GTG GGG TGG AAT GAC				
R H P L Y V D F S D V G W N D				
150	165	180		
TGG ATC GTT GCA CCG CCG GGG TAT CAT GCC TTT TAC TGC CAT GGG				
W I V A P P G Y H A F Y C H G				
195	210	225		
GAG TGC CCT TTT CCC CTG GCC GAT CAC CTT AAC TCC ACG AAT CAT				
E C P F P L A D H L N S T N H				
240	255	270		
GCC ATT CTC CAA ACT CTG GTC AAC TCA GTT AAC TCT AAG ATT CCC				
A I V Q T L V N S V N S K I P				
385	300	315		
AAG GCA TGC TGT GTC CCA ACA GAG CTC AGC GCC ATC TCC ATG CTG				
K A C C V P T E L S A I S M L				
330	345	360		
TAC CTT GAT GAG AAT GAG AAG GTG GTA TTA AAG AAC TAT CAG GAC				
Y L D E N E K V V L K N Y Q D				
375	(129)	397	407	
ATG GTT GTC GAG GGT TGT GGG TGT CGT TAGCACAGCA AAATAAAATA				
M V V E G C G C R				
417	427	437	447	457
TAAATATATA	TATATATATA	TTAGAAAAAC	AGCAAAAAAA	TCAAGTTGAC
467	477	487	497	507
ACTTTAATAT	TTCCCAATGA	AGACTTTATT	TATGGAATGG	AATGGAGAAA
517	527	537	547	557
AAGAAAAACA	CAGCTATTTT	GAAAACTATA	TTTATATCTA	CCGAAAAGAA
567	577	587		
GTTGGGAAAA	CAAATATTTT	AATCAGAGAA	TTATT	

### C. bBMP-3

Probes consisting of pools of oligonucleotides are designed on the basis of the amino acid sequences of the tryptic Fragments 9 (Probe #3), 10 (Probe #2), and 11 (Probe #1), and synthesized on an automated DNA synthesizer.

Probe #1: A C N G T C A T [A/G] T T N G G [A/G] T A

Probe #2: C A [A/G] T A [A/G] T A N G C [A/G] T C [A/G] A A

Probe #3: T G [A/G/T] A T N G T N G C [A/G] T G [A/G] T T

A recombinant bovine genomic library constructed in EMBL3 is screened by the TMAC hybridization procedure detailed above in part A. 400,000 recombinants are screened in duplicate with Probe #1 which has been labeled with  $^{32}\text{P}$ . All recombinants which hybridized to this probe are replated for secondaries. Triplicate nitrocellulose replicas are made of the secondary plates, and amplified as described. The three sets of filters are hybridized to Probes #1, #2 and #3, again under TMAC conditions. One clone, lambda bP-819, hybridizes to all three probes and is plaque purified and DNA is isolated from a plate lysate. Bacteriophage lambda bP-819 was deposited with the ATCC on June 16, 1987 under accession number 40344. This bP-819 clone encodes the bovine bone growth factor designated bBMP-3.

The region of bP-819 which hybridizes to Probe #2 is localized and sequenced. The partial DNA and derived amino acid sequences of this region are shown in Table IVA. The amino acid sequences corresponding to tryptic Fragments 10 and 12 are underlined. The first underlined sequence corresponds to Fragment 12 while the second corresponds to Fragment 10. This region of bP-819, therefore, which hybridizes to Probe #2 encodes at least 111 amino acids. This amino acid sequence is encoded by the DNA sequence from nucleotide #414 through #746.

TABLE IV. A.

383	393	403	413	(1)	428
GAGGAGGAAG	CGGTCTAOGG	GGGTCCCTTCT	GOCTCTGCAG	AAC AAT GAG CTT OCT GGG GCA	Asn Asn Glu Leu Pro Gly Ala
443	458	473	488		
GAA TAT CAG TAC AAG GAG GAT GAA GTA TGG GAG GAG AGG AAG OCT TAC AAG ACT	Glu Tyr Gln Tyr Lys Glu Asp Glu Val Trp Glu Glu Arg Lys Pro Tyr Lys Thr				
503	518	533			
CTT CAG ACT CAG CCC CCT GAT AAG AGT AAG AAC AAA AAG AAA CAG AGG AAG GGA	Leu Gln Thr Gln Pro Pro Asp Lys Ser Lys Asn Lys Lys Lys Gln Arg Lys Gly				
548	563	578	593		
CCT CAG CAG AAG AGT CAG ACG CTC CAG TTT GAT GAA CAG ACC CTG AAG AAG GCA	Pro Gln Gln Lys Ser Gln Thr Leu Gln Phe Asp Glu Gln Thr Leu Lys Lys Ala				
608	623	638			
AGA AGA AAG CAA TGG ATT GAA CCC CGG AAT TGT GCC AGA CCG TAC CTT AAA GTG	Arg Arg Lys Gln Trp Ile Glu Pro Arg Asn Cys Ala Arg Arg Tyr Leu Lys <u>Val</u>				
653	668	683	698		
GAC TTC GCA GAT ATT GGC TGG AGC GAA TGG ATT ATT TCC CCC AAG TCC TTC GAT	<u>Asp Phe Ala Asp Ile Gly Trp Ser Glu Trp Ile Ile Ser Pro Lys Ser Phe Asp</u>				
713	728	743 (111)	756		
GCC TAT TAC TGC TCC GGA GCG TGC CAG TTC CCC ATG CCA AAG GTAGCCATTG	<u>Ala Tyr Tyr Cys Ser Gly Ala Cys Gln Phe Pro MET Pro Lys</u>				
766	776	786			
TTTTTTGTCC	TGTCCCTCCC	ATTTCATAG			



The region of bP-819 which hybridizes to Probe #1 and #3 is localized and sequenced. The partial DNA and derived amino acid sequences of this region are shown in Table IVB. The amino acid sequences corresponding to tryptic Fragments 9 and 11 are underlined. The first underlined sequence corresponds to Fragment 9 while the second underlined sequence corresponds to Fragment 11. The peptide sequence of this region of bP-819 which hybridizes to Probe #1 and #3 is 64 amino acids in length encoded by nucleotide #305 through #493 of Table IVB. The arginine residue encoded by the AGA triplet is presumed to be the carboxy-terminus of the protein based on the presence of a stop codon (TAA) adjacent to it. The nucleic acid sequence preceding the couplet TC (positions 305-306) is presumed to be an intron (non-coding sequence) based on the presence of a consensus acceptor sequence (i.e. a pyrimidine-rich stretch, TTCTCCCTTTTCGTTTCCT, followed by AG) and the presence of a stop rather than a basic residue in the appropriate position of the derived amino acid sequence.

bBMP-3 is therefore characterized by the DNA and amino acid sequence of Table IV A and Table IV B. The peptide sequence of this clone is 175 amino acids in length and is encoded by the DNA sequence from nucleotide #414 through nucleotide #746 of Table IV A and nucleotide #305 through nucleotide #493 of Table IV B.

TABLE IV. B.

284	294	304	(112)	319
CTA	CTT	CTG	CTT	CTG
CTA	CTT	CTG	CTT	CTG
Ser	Leu	Lys	Pro	Ser
Asn	His	Ala	Thr	
334	349	364	379	
ATC	CAG	AGT	ATA	GTG
AGA	GCT	GTG	GGG	GTC
GTC	GTC	CCT	GGA	ATC
CCC	GAG	CCT	TGC	
Ile	Gln	Ser	Ile	Val
Arg	Ala	Val	Gly	Val
Val	Val	Pro	Gly	Ile
Pro	Glu	Pro	Cys	
394	409	424	439	
TGT	GTG	CCA	GAA	AAG
ATG	TOC	TCA	CTC	AGC
ATC	TTA	TTC	TTT	GAT
GAA	AAC	AAG		
Cys	Val	Pro	Glu	Lys
MET	Ser	Ser	Leu	Ser
Ile	Leu	Phe	Phe	Asp
Glu	Asn	Lys		
454	469	484	(175)	
AAT	GTG	GTA	CIT	AAA
GTA	TAT	CCA	AAC	ATG
ACA	GTA	GAG	TCT	TGT
GCT	TGC	AGA		
Asn	Val	Val	Leu	Lys
Val	Tyr	Pro	Asn	MET
Thr	Val	Glu	Ser	Cys
Ala	Cys	Arg		
503	513	523	533	
TAA	CTG	GGT	G	
AAG	AAC	T	CAT	
CTG	GAT	GCT	T	
AACT	CAAT	CG		

EXAMPLE VHuman Bone Inductive FactorsA. hBMP-1

Because the bovine and human bone growth factor genes are presumed to be significantly homologous, the bovine bBMP-1 DNA sequence of Table II (or portions thereof) is used as a probe to screen a human genomic library. The 800bp EcoRI fragment of the bovine genomic clone is labeled with  $^{32}\text{P}$  by nick-translation. A human genomic library (Toole et al., *supra*) is plated on 20 plates at 40,000 recombinants per plate. Duplicate nitrocellulose filter replicas are made of each plate and hybridized to the nick-translated probe in 5 X SSC, 5 X Denhardt's, 100ug/ml denatured salmon sperm DNA, 0.1% SDS (the standard hybridization solution) at 50 degrees centigrade for approximately 14 hours. The filters are then washed in 1 X SSC, 0.1% SDS at 50 degrees centigrade and subjected to autoradiography. Five duplicate positives are isolated and plaque purified. DNA is obtained from a plate lysate of one of these recombinant bacteriophage, designated LP-H1. LP-H1 was deposited with the ATCC on March 6, 1987 under accession number 40311. This clone encodes at least a portion of the human genomic bone growth factor called hBMP-1. The hybridizing region of LP-H1 is localized to a 2.5kb XbaI/HindIII restriction fragment.

The partial DNA sequence and derived amino acid sequence of lambda LP-H1 are shown below in Table V. The peptide sequence from this clone is 37 amino acids in length and is encoded by the DNA sequence from nucleotide #3440 through nucleotide #3550. The coding sequence of Table V is flanked by approximately 28 nucleotides (a presumptive 5' noncoding sequence) as well as approximately 19 nucleotides (a presumptive 3' noncoding sequence). A comparison of the bBMP-1 sequence of Table II with the hBMP-1 genomic sequence of Table V indicates the significant homology between the two.

Because the size of coding regions and the positions

of noncoding regions is generally conserved in homologous genes of different species, the locations of the coding and noncoding regions of the bone inductive factor genes may be identified. Regions of homology between the two species' genes, flanked by RNA processing signals at homologous sites, indicate a coding region.



TABLE V

3419	3429	3439	(1)	3454
CAGCCCTGGC	TTCCTCTTTT	CTCTTCTAGCT	GCC TTT CTT GGG GAC ATT GCC CTG GAC	
			Ala Phe Leu Gly Asp Ile Ala Leu Asp	
3469	3484	3499	3514	
GAA GAG GAC CTG AGG GCC TTC CAG GTA CAG CAG GCT GTG GAT CTC AGA OGG CAC				
Glu Glu Asp Leu Arg Ala Phe Gln Val Gln Gln Ala Val Asp Leu Arg Arg His				
3529	3544	(37)	3560	3570
ACA GCT CGT AAG TCC TCC ATC AAA GCT GCA GGTAAGCCGG GTGCCAATGG				
Thr Ala Arg Lys Ser Ser Ile Lys Ala Ala				

A probe specific for the human coding sequence given in Table V is used to identify a human cell line or tissue which synthesizes bone inductive factor. The probe is made according to the following method. Two oligonucleotides having the following sequences:

(a) GGGAATTCTGCCTTTCTTGGGGACATTGCCCTGGACGAAGAGGACCTGAG

(b) CGGGATCCGTCTGAGATCCACAGCCTGCTGTACCTGGAAGGCCCTCAGG

are synthesized on an automated synthesizer, annealed, extended using the Klenow fragment of E. coli DNA polymerase I, digested with the restriction enzymes Eco RI and Bam HI, and inserted into an M13 vector. A single-stranded  $^{32}\text{P}$ -labeled probe is then from template preparation of this subclone by standard techniques. Polyadenylated RNAs from various cell and tissue sources are electrophoresed on formaldehyde-agarose gels and transferred to nitrocellulose by the method of Toole et al., supra. The probe is then hybridized to the nitrocellulose blot in 50% formamide, 5 X SSC, 0.1% SDS, 40 mM sodium phosphate pH 6.5, 100 ug/ml denatured salmon sperm DNA, and 5 mM vanadyl ribonucleosides at 42° C overnight and washed at 65° C in 0.2 X SSC, 0.1% SDS. Following autoradiography, the lane containing RNA from the human osteosarcoma cell line U-2 OS contains hybridizing bands corresponding to RNA species of approximately 4.3 and 3.0 kb.

cDNA is synthesized from U-2 OS polyadenylated RNA and cloned into lambda gt10 by established techniques (Toole et al., supra). 20,000 recombinants from this library are plated on each of 50 plates. Duplicate nitrocellulose replicas are made of the plates. The above described oligonucleotides are kinased with  $^{32}\text{P}$ -gamma-ATP and hybridized to the two sets of replicas at 55° centigrade in standard hybridization solution overnight. The filters are then washed in 1 X SSC, 0.1% SDS at 55° centigrade and subjected to autoradiography. One duplicate positive, designated lambda U2OS-1, is plaque purified. Lambda U2OS-1 was deposited with the ATCC on June 16, 1987 under accession number 40343.

The entire nucleotide sequence and derived amino acid sequence of the insert of lambda U2OS-1 is given in Table VI. This cDNA clone encodes a Met followed by a hydrophobic leader sequence characteristic of a secreted protein, and contains a stop codon at nucleotide positions 2226 - 2228. This clone contains an open reading frame of 2190bp, encoding a protein of 730 amino acids with a molecular weight of 83kd based on this amino acid sequence. The clone contains sequence identical to the coding region given in Table V. This protein is contemplated to represent a primary translation product which is cleaved upon secretion to produce the hBMP-1 protein. This clone is therefore a cDNA for hBMP-1 corresponding to human gene fragment contained in the genomic hBMP-1 sequence lambda LP-H1. It is noted that amino acids #550 to #590 of BMP-1 are homologous to epidermal growth factor and the "growth factor" domains of Protein C, Factor X and Factor IX.

TABLE VI

10	20	30	(1)	50
CTAGAGGCOG	CTTCOCTOGC	OGGOGOOOOG	CCAGC	ATG CCC GGC GTG GOC OGC CTG COG
				MET Pro Gly Val Ala Arg Leu Pro
65	80	95	110	
CTG CTG CTC GGG CTG CTG CTG CTC COG OGT CCC GGC OGG COG CTG GAC TTG GOC				
Leu Leu Leu Gly Leu Leu Leu Leu Pro Arg Pro Gly Arg Pro Leu Asp Leu Ala				
125	140	155		
GAC TAC ACC TAT GAC CTG GOG GAG GAG GAC GAC TCG GAG CCC CTC AAC TAC AAA				
Asp Tyr Thr Tyr Asp Leu Ala Glu Glu Asp Asp Ser Glu Pro Leu Asn Tyr Lys				
170	185	200	215	
GAC CCC TGC AAG GOG GCT GOC TTT CTT GGG GAC ATT GCC CTG GAC GAA GAG GAC				
Asp Pro Cys Lys Ala Ala Ala Phe Leu Gly Asp Ile Ala Leu Asp Glu Glu Asp				
230	245	260	275	
CTG AGG GOC TTC CAG GTA CAG CAG GCT GTG GAT CTC AGA OGG CAC ACA GCT OGT				
Leu Arg Ala Phe Gln Val Gln Gln Ala Val Asp Leu Arg Arg His Thr Ala Arg				
290	305	320		
AAG TCC TCC ATC AAA GCT GCA GTT CCA GGA AAC ACT TCT ACC CCC AGC TGC CAG				
Lys Ser Ser Ile Lys Ala Ala Val Pro Gly Asn Thr Ser Thr Pro Ser Cys Gln				
335	350	365	380	
AGC ACC AAC GGG CAG CCT CAG AGG GGA GOC TGT GGG AGA TGG AGA GGT AGA TCC				
Ser Thr Asn Gly Gln Pro Gln Arg Gly Ala Cys Gly Arg Trp Arg Gly Arg Ser				
395	410	425		
OGT AGC OGG OGG GOG GOG ACG TOC OGA CCA GAG OGT GTG TGG CCC GAT GGG GTC				
Arg Ser Arg Arg Ala Ala Thr Ser Arg Pro Glu Arg Val Trp Pro Asp Gly Val				
440	455	470	485	
ATC CCC TTT GTC ATT GGG GGA AAC TTC ACT GGT AGC CAG AGG GCA GTC TTC OGG				
Ile Pro Phe Val Ile Gly Gly Asn Phe Thr Gly Ser Gln Arg Ala Val Phe Arg				
500	515	530	545	
CAG GOC ATG AGG CAC TGG GAG AAG CAC ACC TGT GTC ACC TTC CTG GAG OGC ACT				
Gln Ala MET Arg His Trp Glu Lys His Thr Cys Val Thr Phe Leu Glu Arg Thr				
560	575	590		
GAC GAG GAC AGC TAT ATT GTG TTC ACC TAT OGA CCT TGC GGG TGC TGC TOC TAC				
Asp Glu Asp Ser Tyr Ile Val Phe Thr Tyr Arg Pro Cys Gly Cys Cys Ser Tyr				
605	620	635	650	
GTG GGT OGC OGC GGC GGG GGC CCC CAG GOC ATC TOC ATC GGC AAG AAC TGT GAC				
Val Gly Arg Arg Gly Gly Gly Pro Gln Ala Ile Ser Ile Gly Lys Asn Cys Asp				

665                      680                      695  
 AAG TTC GGC ATT GTG GTC CAC GAG CTG GGC CAC GTC GTC GGC TTC TGG CAC GAA  
 Lys Phe Gly Ile Val Val His Glu Leu Gly His Val Val Gly Phe Trp His Glu  
  
 710                      725                      740                      755  
 CAC ACT OGG CCA GAC OGG GAC OGC CAC GTT TOC ATC GTT OGT GAG AAC ATC CAG  
 His Thr Arg Pro Asp Arg Asp Arg His Val Ser Ile Val Arg Glu Asn Ile Gln  
  
 770                      785                      800                      815  
 CCA GGG CAG GAG TAT AAC TTC CTG AAG ATG GAG CCT CAG GAG GTG GAG TOC CTG  
 Pro Gly Gln Glu Tyr Asn Phe Leu Lys MET Glu Pro Gln Glu Val Glu Ser Leu  
  
 830                      845                      860  
 GGG GAG ACC TAT GAC TTC GAC AGC ATC ATG CAT TAC GCT OGG AAC ACA TTC TOC  
 Gly Glu Thr Tyr Asp Phe Asp Ser Ile MET His Tyr Ala Arg Asn Thr Phe Ser  
  
 875                      890                      905                      920  
 AGG GGC ATC TTC CTG GAT ACC ATT GTC CCC AAG TAT GAG GTG AAC GGG GTG AAA  
 Arg Gly Ile Phe Leu Asp Thr Ile Val Pro Lys Tyr Glu Val Asn Gly Val Lys  
  
 935                      950                      965  
 CCT CCC ATT GGC CAA AGG ACA OGG CTC AGC AAG GGG GAC ATT GCC CAA GCC OGC  
 Pro Pro Ile Gly Gln Arg Thr Arg Leu Ser Lys Gly Asp Ile Ala Gln Ala Arg  
  
 980                      995                      1010                      1025  
 AAG CTT TAC AAG TGC CCA GCC TGT GGA GAG ACC CTG CAA GAC AGC ACA GGC AAC  
 Lys Leu Tyr Lys Cys Pro Ala Cys Gly Glu Thr Leu Gln Asp Ser Thr Gly Asn  
  
 1040                      1055                      1070                      1085  
 TTC TOC TOC CCT GAA TAC CCC AAT GGC TAC TCT GCT CAC ATG CAC TGC GTG TGG  
 Phe Ser Ser Pro Glu Tyr Pro Asn Gly Tyr Ser Ala His MET His Cys Val Trp  
  
 1100                      1115                      1130  
 OGC ATC TCT GTC ACA CCC GGG GAG AAG ATC ATC CTG AAC TTC ACG TOC CTG GAC  
 Arg Ile Ser Val Thr Pro Gly Glu Lys Ile Ile Leu Asn Phe Thr Ser Leu Asp  
  
 1145                      1160                      1175                      1190  
 CTG TAC OGC AGC OGC CTG TGC TGG TAC GAC TAT GTG GAG GTC OGA GAT GGC TTC  
 Leu Tyr Arg Ser Arg Leu Cys Trp Tyr Asp Tyr Val Glu Val Arg Asp Gly Phe  
  
 1205                      1220                      1235  
 TGG AGG AAG GOG CCC CTC OGA GGC OGC TTC TGC GGG TOC AAA CTC CCT GAG CCT  
 Trp Arg Lys Ala Pro Leu Arg Gly Arg Phe Cys Gly Ser Lys Leu Pro Glu Pro  
  
 1250                      1265                      1280                      1295  
 ATC GTC TOC ACT GAC AGC OGC CTC TGG GTT GAA TTC OGC AGC AGC AGC AAT TGG  
 Ile Val Ser Thr Asp Ser Arg Leu Trp Val Glu Phe Arg Ser Ser Ser Asn Trp  
  
 1310                      1325                      1340                      1355  
 GTT GGA AAG GGC TTC TTT GCA GTC TAC GAA GCC ATC TGC GGG GGT GAT GTG AAA  
 Val Gly Lys Gly Phe Phe Ala Val Tyr Glu Ala Ile Cys Gly Gly Asp Val Lys



1370					1385					1400									
AAG	GAC	TAT	GGC	CAC	ATT	CAA	TOG	CCC	AAC	TAC	CCA	GAC	GAT	TAC	OGG	CCC	AGC		
Lys	Asp	Tyr	Gly	His	Ile	Gln	Ser	Pro	Asn	Tyr	Pro	Asp	Asp	Tyr	Arg	Pro	Ser		
1415					1430					1445					1460				
AAA	GTC	TGC	ATC	TGG	OGG	ATC	CAG	GTG	TCT	GAG	GGC	TTC	CAC	GTG	GGC	CTC	ACA		
Lys	Val	Cys	Ile	Trp	Arg	Ile	Gln	Val	Ser	Glu	Gly	Phe	His	Val	Gly	Leu	Thr		
1475					1490					1505									
TTC	CAG	TOC	TTT	GAG	ATT	GAG	OGC	CAC	GAC	AGC	TGT	GCC	TAC	GAC	TAT	CTG	GAG		
Phe	Gln	Ser	Phe	Glu	Ile	Glu	Arg	His	Asp	Ser	Cys	Ala	Tyr	Asp	Tyr	Leu	Glu		
1520					1535					1550					1565				
GTG	OGC	GAC	GGG	CAC	AGT	GAG	AGC	AGC	ACC	CTC	ATC	GGG	OGC	TAC	TGT	GGC	TAT		
Val	Arg	Asp	Gly	His	Ser	Glu	Ser	Ser	Thr	Leu	Ile	Gly	Arg	Tyr	Cys	Gly	Tyr		
1580					1595					1610					1625				
GAG	AAG	CCT	GAT	GAC	ATC	AAG	AGC	ACG	TCC	AGC	OGC	CTC	TGG	CTC	AAG	TTC	GTC		
Glu	Lys	Pro	Asp	Asp	Ile	Lys	Ser	Thr	Ser	Ser	Arg	Leu	Trp	Leu	Lys	Phe	Val		
1640					1655					1670									
TCT	GAC	GGG	TCC	ATT	AAC	AAA	GCG	GGC	TTT	GCC	GTC	AAC	TTT	TTC	AAA	GAG	GTG		
Ser	Asp	Gly	Ser	Ile	Asn	Lys	Ala	Gly	Phe	Ala	Val	Asn	Phe	Phe	Lys	Glu	Val		
1685					1700					1715					1730				
GAC	GAG	TGC	TCT	OGG	CCC	AAC	OGC	GGG	GGC	TGT	GAG	CAG	OGG	TGC	CTC	AAC	ACC		
Asp	Glu	Cys	Ser	Arg	Pro	Asn	Arg	Gly	Gly	Cys	Glu	Gln	Arg	Cys	Leu	Asn	Thr		
1745					1760					1775									
CTG	GGC	AGC	TAC	AAG	TGC	AGC	TGT	GAC	CCC	GGG	TAC	GAG	CTG	GCC	CCA	GAC	AAG		
Leu	Gly	Ser	Tyr	Lys	Cys	Ser	Cys	Asp	Pro	Gly	Tyr	Glu	Leu	Ala	Pro	Asp	Lys		
1790					1805					1820					1835				
OGC	OGC	TGT	GAG	GCT	GCT	TGT	GGC	GGA	TTC	CTC	AOC	AAG	CTC	AAC	GGC	TCC	ATC		
Arg	Arg	Cys	Glu	Ala	Ala	Cys	Gly	Gly	Phe	Leu	Thr	Lys	Leu	Asn	Gly	Ser	Ile		
1850					1865					1880					1895				
ACC	AGC	CCG	GGC	TGG	CCC	AAG	GAG	TAC	CCC	CCC	AAC	AAG	AAC	TGC	ATC	TGG	CAG		
Thr	Ser	Pro	Gly	Trp	Pro	Lys	Glu	Tyr	Pro	Pro	Asn	Lys	Asn	Cys	Ile	Trp	Gln		
1910					1925					1940									
CTG	GTG	GCC	CCC	ACC	CAG	TAC	OGC	ATC	TCC	CTG	CAG	TTT	GAC	TTC	TTT	GAG	ACA		
Leu	Val	Ala	Pro	Thr	Gln	Tyr	Arg	Ile	Ser	Leu	Gln	Phe	Asp	Phe	Phe	Glu	Thr		
1955					1970					1985					2000				
GAG	GGC	AAT	GAT	GTG	TGC	AAG	TAC	GAC	TTC	GTG	GAG	GTG	OGC	AGT	GGA	CTC	ACA		
Glu	Gly	Asn	Asp	Val	Cys	Lys	Tyr	Asp	Phe	Val	Glu	Val	Arg	Ser	Gly	Leu	Thr		
2015					2030					2045									
GCT	GAC	TCC	AAG	CTG	CAT	GGC	AAG	TTC	TGT	GGT	TCT	GAG	AAG	CCC	GAG	GTC	ATC		
Ala	Asp	Ser	Lys	Leu	His	Gly	Lys	Phe	Cys	Gly	Ser	Glu	Lys	Pro	Glu	Val	Ile		

2060                      2075                      2090                      2105  
 ACC TOC CAG TAC AAC AAC ATG OGC GTG GAG TTC AAG TOC GAC AAC ACC GTG TOC  
 Thr Ser Gln Tyr Asn Asn MET Arg Val Glu Phe Lys Ser Asp Asn Thr Val Ser  
  
                     2120                      2135                      2150                      2165  
 AAA AAG GGC TTC AAG GOC CAC TTC TTC TCA GAA AAG AGG CCA GCT CTG CAG CCC  
 Lys Lys Gly Phe Lys Ala His Phe Phe Ser Glu Lys Arg Pro Ala Leu Gln Pro  
  
                     2180                      2195                      2210  
 CCT OGG GGA OGC OCC CAC CAG CTC AAA TTC OGA GTG CAG AAA AGA AAC OGG ACC  
 Pro Arg Gly Arg Pro His Gln Leu Lys Phe Arg Val Gln Lys Arg Asn Arg Thr  
 (730)  
                     2225                      2235                      2245                      2255                      2265                      2275                      2285  
 CCC CAG TGAGGCTGC CAGGCTOCC GGACCCCTTG TTA CTCAGGA ACCTCACCTT GGACGGAATG  
 Pro Gln  
  
                     2295                      2305                      2315                      2325                      2335                      2345                      2355  
 GGATGGGGGC TTOGGTGCCC ACCAACCCCC CACCTCCACT CTGCCATTCC GGCCACCTC CCTCTGGCOG  
  
                     2365                      2375                      2385                      2395                      2405                      2415                      2425  
 GACAGAACTG GTGCTCTCTT CTCCCCACTG TGCCOGTGCG OGGACOGGGG ACCCTTCCCC GTGCCCTACC  
  
                     2435                      2445                      2455                      2465                      2475                      2485                      2495  
 -CCCTOCCATT-TTGATGGTGT-CTGTGACATT-TCCTGTTGTG-AAGTAAAAGA GGGACCCCTG CGTCTGCTT

CTAGA

### B. hBMP-2: Class I and II

The HindIII-SacI bovine genomic bBMP-2 fragment described in Example IV B. is subcloned into an M13 vector. A  $^{32}\text{P}$ -labeled single-stranded DNA probe is made from a template preparation of this subclone. This probe is used to screen polyadenylated RNAs from various cell and tissue sources as described above in part A. A hybridizing band corresponding to an mRNA species of approximately 3.8 kb is detected in the lane containing RNA from the human cell line U-2 OS. The HindIII-SacI fragment is labeled with  $^{32}\text{P}$  by nick translation and used to screen the nitrocellulose filter replicas of the above-described U-2 OS cDNA library by hybridization in standard hybridization buffer at  $65^{\circ}$  overnight followed by washing in 1 X SSC, 0.1% SDS at  $65^{\circ}$ . Twelve duplicate positive clones are picked and replated for secondaries. Duplicate nitrocellulose replicas are made of the secondary plates and both sets hybridized to the bovine genomic probe as the primary screening was performed. One set of filters is then washed in 1 X SSC, 0.1% SDS; the other in 0.1 X SSC, 0.1% SDS at  $65^{\circ}$ .

Two classes of hBMP-2 cDNA clones are evident based on strong (4 recombinants) or weak (7 recombinants) hybridization signals under the more stringent washing conditions (0.1 X SSC, 0.1% SDS). All 11 recombinant bacteriophage are plaque purified, small scale DNA preparations made from plate lysates of each, and the inserts subcloned into pSP65 and into M13 for sequence analysis. Sequence analysis of the strongly hybridizing clones designated hBMP-2 Class I (also known as BMP-2) indicates that they have extensive sequence homology with the sequence given in Table III. These clones are therefore cDNA encoding the human equivalent of the protein encoded by the bBMP-2 gene whose partial sequence is given in Table III. Sequence analysis of the weakly hybridizing recombinants designated hBMP-2 Class II (also known as BMP-4) indicates that they are also quite homologous

with the sequence given in Table III at the 3' end of their coding regions, but less so in the more 5' regions. Thus they encode a human protein of similar, though not identical, structure to that above.

Full length hBMP-2 Class I cDNA clones are obtained in the following manner. The 1.5 kb insert of one of the Class II subclones (II-10-1) is isolated and radioactively labeled by nick-translation. One set of the nitrocellulose replicas of the U-2 OS cDNA library screened above (50 filters, corresponding to 1,000,000 recombinant bacteriophage) are rehybridized with this probe under stringent conditions (hybridization at 65° in standard hybridization buffer; washing at 65° in 0.2 X SSC, 0.1% SDS). All recombinants which hybridize to the bovine genomic probe which do not hybridize to the Class II probe are picked and plaque purified (10 recombinants). Plate stocks are made and small scale bacteriophage DNA preparations made. After subcloning into M13, sequence analysis indicates that 4 of these represent clones which overlap the original Class I clone. One of these, lambda U2OS-39, contains an approximately 1.5 kb insert and was deposited with the ATCC on June 16, 1987 under accession number 40345. The partial DNA sequence (compiled from lambda U2OS-39 and several other hBMP-2 Class I cDNA recombinants) and derived amino acid sequence are shown below in Table VII. Lambda U2OS-39 is expected to contain all of the nucleotide sequence necessary to encode the entire human counterpart of the protein BMP-2 Class II encoded by the bovine gene segment whose partial sequence is presented in Table III. This human cDNA hBMP-2 Class II contains an open reading frame of 1188 bp, encoding a protein of 396 amino acids. This protein of 396 amino acids has a molecular weight of 45kd based on this amino acid sequence. It is contemplated that this sequence represents the primary translation product. The protein is preceded by a 5' untranslated region of 342 bp with stop codons in all frames.

The 13 bp region preceding this 5' untranslated region represents a linker used in the cDNA cloning procedure.



TABLE VII

10	20	30	40	50	60	70
GTOGACTCTA	GAGTGTGTGT	CAGCACTTGG	CTGGGGACTT	CTTGAACCTG	CAGGGAGAAT	AACTTGOGCA
80	90	100	110	120	130	140
CCCCACTTTG	OGCOGGTGGC	TTTGCCCCAG	OGGAGCCTGC	TTOGCCATCT	COGAGCCCCA	COGCCCCCTCC
150	160	170	180	190	200	210
ACTOCTOGGC	CTTGCCCOGAC	ACTGAGAOGC	TGTTCCOCAGC	GTGAAAAGAG	AGACTGOGOG	GCOGGCACCC
220	230	240	250	260	270	280
GGGAGAAGGA	GGAGGCAAAG	AAAAGGAAOG	GACATTGGGT	CCMTGOGCCA	GGTCCMTTGA	CCAGAGTTTT
290	300	310	320	330	340	350
TCCATGTGGA	CGCTCTTTCA	ATGGAOGTGT	CCCOGOGTGC	TTCTTAGAOG	GACTGOGGTC	TOCTAAAGGT
<hr/>						
(1)	370	385	400			
OGACC	ATG GTG GGC GGG ACC OGC TGT CTT CTA GCG TTG CTG CTT CCC CAG GTC					
MET	Val Ala Gly Thr Arg Cys Leu Leu Ala Leu Leu Leu Pro Gln Val					
415	430	445				
CTC CTG GGC GGC GCG GCT GGC CTC GTT CCG GAG CTG GGC OGC AGG AAG TTC GCG						
Leu Leu Gly Gly Ala Ala Gly Leu Val Pro Glu Leu Gly Arg Arg Lys Phe Ala						
460	475	490	505			
GCG GCG TOG TOG GGC OGC CCC TCA TCC CAG CCC TCT GAC GAG GTC CTG AGC GAG						
Ala Ala Ser Ser Gly Arg Pro Ser Ser Gln Pro Ser Asp Glu Val Leu Ser Glu						
520	535	550	565			
TTC GAG TTG OGG CTG CTC AGC ATG TTC GGC CTG AAA CAG AGA CCC ACC CCC AGC						
Phe Glu Leu Arg Leu Leu Ser MET Phe Gly Leu Lys Gln Arg Pro Thr Pro Ser						
580	595	610				
AGG GAC GGC GTG GTG CCC CCC TAC ATG CTA GAC CTG TAT OGC AGG CAC TOG GGT						
Arg Asp Ala Val Val Pro Pro Tyr MET Leu Asp Leu Tyr Arg Arg His Ser Gly						
625	640	655	670			
CAG CCG GGC TCA CCC GCC CCA GAC CAC OGG TTG GAG AGG GCA GCC AGC CGA GCC						
Gln Pro Gly Ser Pro Ala Pro Asp His Arg Leu Glu Arg Ala Ala Ser Arg Ala						
685	700	715				
AAC ACT GTG OGC AGC TTC CAC CAT GAA GAA TCT TTG GAA GAA CTA CCA GAA ACG						
Asn Thr Val Arg Ser Phe His His Glu Glu Ser Leu Glu Glu Leu Pro Glu Thr						

730	745	760	775
AGT GGG AAA ACA ACC OGG AGA TTC TTC TTT AAT TTA AGT TCT ATC CCC ACG GAG			
Ser Gly Lys Thr Thr Arg Arg Phe Phe Phe Asn Leu Ser Ser Ile Pro Thr Glu			
790	805	820	835
GAG TTT ATC ACC TCA GCA GAG CTT CAG GTT TTC OGA GAA CAG ATG CAA GAT GCT			
Glu Phe Ile Thr Ser Ala Glu Leu Gln Val Phe Arg Glu Gln MET Gln Asp Ala			
850	865	880	
TTA GGA AAC AAT AGC AGT TTC CAT CAC CGA ATT AAT ATT TAT GAA ATC ATA AAA			
Leu Gly Asn Asn Ser Ser Phe His His Arg Ile Asn Ile Tyr Glu Ile Ile Lys			
895	910	925	940
CCT GCA ACA GGC AAC TCG AAA TTC CCC GTG ACC AGT CTT TTG GAC ACC ACG TTG			
Pro Ala Thr Ala Asn Ser Lys Phe Pro Val Thr Ser Leu Leu Asp Thr Arg Leu			
955	970	985	
GTG AAT CAG AAT GCA AGC AGG TGG GAA AGT TTT GAT GTC ACC CCC GCT GTG ATG			
Val Asn Gln Asn Ala Ser Arg Trp Glu Ser Phe Asp Val Thr Pro Ala Val MET			
1000	1015	1030	1045
CGG TGG ACT GCA CAG GGA CAC GCC AAC CAT GGA TTC GTG GTG GAA GTG GCC CAC			
Arg Trp Thr Ala Gln Gly His Ala Asn His Gly Phe Val Val Glu Val Ala His			
1060	1075	1090	1105
TTG GAG GAG AAA CAA GGT GTC TCC AAG AGA CAT GTT AGG ATA AGC AGG TCT TTG			
Leu Glu Glu Lys Gln Gly Val Ser Lys Arg His Val Arg Ile Ser Arg Ser Leu			
1120	1135	1150	
CAC CAA GAT GAA CAC AGC TGG TCA CAG ATA AGG CCA TTG CTA GTA ACT TTT GGC			
His Gln Asp Glu His Ser Trp Ser Gln Ile Arg Pro Leu Leu Val Thr Phe Gly			
1165	1180	1195	1210
CAT GAT GGA AAA GGG CAT CCT CTC CAC AAA AGA GAA AAA CGT CAA GCC AAA CAC			
His Asp Gly Lys Gly His Pro Leu His Lys Arg Glu Lys Arg Gln Ala Lys His			
1225	1240	1255	
AAA CAG OGG AAA OGC CTT AAG TCC AGC TGT AAG AGA CAC CCT TTG TAC GTG GAC			
Lys Gln Arg Lys Arg Leu Lys Ser Ser Cys Lys Arg His Pro Leu Tyr Val Asp			
1270	1285	1300	1315
TTC AGT GAC GTG GGG TGG AAT GAC TGG ATT GTG GCT CCC CCG GGG TAT CAC GCC			
Phe Ser Asp Val Gly Trp Asn Asp Trp Ile Val Ala Pro Pro Gly Tyr His Ala			
1330	1345	1360	1375
TTT TAC TGC CAC GGA GAA TGC CCT TTT CCT CTG GCT GAT CAT CTG AAC TCC ACT			
Phe Tyr Cys His Gly Glu Cys Pro Phe Pro Leu Ala Asp His Leu Asn Ser Thr			
1390	1405	1420	
AAT CAT GCC ATT GTT CAG ACG TTG GTC AAC TCT GTT AAC TCT AAG ATT CCT AAG			
Asn His Ala Ile Val Gln Thr Leu Val Asn Ser Val Asn Ser Lys Ile Pro Lys			

1435 1450 1465 1480  
GCA TGC TGT GTC CCG ACA GAA CTC AGT GCT ATC TCG ATG CTG TAC CTT GAC GAG  
Ala Cys Cys Val Pro Thr Glu Leu Ser Ala Ile Ser MET Leu Tyr Leu Asp Glu

1495 1510 1525  
AAT GAA AAG GTT GTA TTA AAG AAC TAT CAG GAC ATG GTT GTG GAG GGT TGT GGG  
Asn Glu Lys Val Val Leu Lys Asn Tyr Gln Asp MET Val Val Glu Gly Cys Gly

1540(396) 1553 1563 1573 1583 1593 1603  
TGT CGC TAGTACAGCA AAATTAAATA CATAAATATA TATATATATA TATATTTTAG AAAAAAGAAA  
Cys=Arg

AAAA:

Full-length hBMP-2 Class II human cDNA clones are obtained in the following manner. The 200 bp EcoRI-SacI fragment from the 5' end of the Class II recombinant II-10-1 is isolated from its plasmid subclone, labeled by nick-translation, and hybridized to a set of duplicate nitrocellulose replicas of the U-2 OS cDNA library (25 filters/set; representing 500,000 recombinants). Hybridization and washing are performed under stringent conditions as described above. 16 duplicate positives are picked and replated for secondaries. Nitrocellulose filter replicas of the secondary plates are made and hybridized to an oligonucleotide which was synthesized to correspond to the sequence of II-10-1 and is of the following sequence:

CGGGCGCTCAGGATACTCAAGACCAGTGCTG

Hybridization is in standard hybridization buffer AT 50° C with washing at 50° in 1 X SSC, 0.1% SDS. 14 recombinant bacteriophage which hybridize to this oligonucleotide are plaque purified. Plate stocks are made and small scale bacteriophage DNA preparations made. After succloning 3 of these into M13, sequence analysis indicates that they represent clones which overlap the original Class II clone. One of these, lambda U20S-3, was deposited with the ATCC under accession number 40342 on June 16, 1987. U20S-3 contains an insert of approximately 1.8 kb. The partial DNA sequence and derived amino acid sequence of U20S-3 are shown below in Table VIII. This clone is expected to contain all of the nucleotide sequence necessary to encode the entire human BMP-2 Class II protein. This cDNA contains an open reading frame of 1224 bp, encoding a protein of 408 amino acids, preceded by a 5' untranslated region of 394 bp with stop codons in all frames, and contains a 3' untranslated region of 308 bp following the in-frame stop codon. The 8 bp region preceding the 5' untranslated region represents a linker used in the cDNA cloning procedure. This protein of 408 amino acids has molecular weight of 47kd and is contemplated to represent the

primary translation product.

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TABLE VIII

10	20	30	40	50	60	70
CTCTAGAGGG	CAGAGGAGGA	GGGAGGGAGG	GAAGGAGOGC	GGAGCCOOGC	COGGAAGCTA	GGTGAGTGIG
80	90	100	110	120	130	140
GCATCOGAGC	TGAGGGAOGC	GAGCCTGAGA	OGCOGCTGCT	GCTCOGGCTG	AGTATCTAGC	TTGTCTCCCC
150	160	170	180	190	200	210
GATGGGATTG	COGTCCAAGC	TATCTOGAGC	CTGCAGOGCC	ACAGTCCCOG	GCCCTOGCCC	AGGTTCACTG
220	230	240	250	260	270	280
CAACOGTTCA	GAGGTCCOCC	GGAGCTGCTG	CTGGOGAGCC	OGCTACTGCA	GGGACCTATG	GAGCCATTCC
290	300	310	320	330	340	350
GTAGTGCCAT	COOGAGCAAC	GCACTGCTGC	AGCTTCOCTG	AGCCTTTCCA	GCAAGTTTGT	TCAAGATTGG
360	370	380	390	400	(1)	
CTGTCAAGAA	TCATGGACTG	TTATTATATG	CCTTGTTTTT	TGTCAAGACA	CC ATG ATT CCT	
					MET Ile Pro	
417		432		447		462
GGT AAC CGA ATG CTG ATG GTC GTT TTA TTA TGC CAA GTC CTG CTA GGA GGC GCG						
Gly Asn Arg MET Leu MET Val Val Leu Leu Cys Gln Val Leu Leu Gly Gly Ala						
477		492		507		
AGC CAT GCT AGT TTG ATA CCT GAG ACG GGG AAG AAA AAA GTC GGC GAG ATT CAG						
Ser His Ala Ser Leu Ile Pro Glu Thr Gly Lys Lys Lys Val Ala Glu Ile Gln						
522	537	552		567		
GGC CAC GCG GGA GGA OGC OGC TCA GGG CAG AGC CAT GAG CTC CTG OGG GAC TTC						
Gly His Ala Gly Gly Arg Arg Ser Gly Gln Ser His Glu Leu Leu Arg Asp Phe						
582		597		612		627
GAG GCG ACA CTT CTG CAG ATG TTT GGG CTG OGC OGC OGC COG CAG CCT AGC AAG						
Glu Ala Thr Leu Leu Gln MET Phe Gly Leu Arg Arg Arg Pro Gln Pro Ser Lys						
642		657		672		
AGT GGC GTC ATT COG GAC TAC ATG OGC GAT CTT TAC OGC CTT CAG TCT GGG GAG						
Ser Ala Val Ile Pro Asp Tyr MET Arg Asp Leu Tyr Arg Leu Gln Ser Gly Glu						
687	702	717		732		
GAG GAG GAA GAG CAG ATC CAC AGC ACT GGT CTT GAG TAT CCT GAG OGC COG GCC						
Glu Glu Glu Glu Gln Ile His Ser Thr Gly Leu Glu Tyr Pro Glu Arg Pro Ala						

747                      762                      777  
 AGC OGG GOC AAC ACC GTG AGG AGC TTC CAC CAC GAA GAA CAT CTG GAG AAC ATC  
 Ser Arg Ala Asn Thr Val Arg Ser Phe His His Glu Glu His Leu Glu Asn Ile

792                      807                      822                      837  
 CCA GGG ACC AGT GAA AAC TCT GCT TTT OGT TTC CTC TTT AAC CTC AGC AGC ATC  
 Pro Gly Thr Ser Glu Asn Ser Ala Phe Arg Phe Leu Phe Asn Leu Ser Ser Ile

852                      867                      882                      897  
 CCT GAG AAC GAG GTG ATC TCC TCT GCA GAG CTT OGG CTC TTC OGG GAG CAG GTG  
 Pro Glu Asn Glu Val Ile Ser Ser Ala Glu Leu Arg Leu Phe Arg Glu Gln Val

912                      927                      942  
 GAC CAG GGC OCT GAT TGG GAA AGG GGC TTC CAC OGT ATA AAC ATT TAT GAG GTT  
 Asp Gln Gly Pro Asp Trp Glu Arg Gly Phe His Arg Ile Asn Ile Tyr Glu Val

957                      972                      987                      1002  
 ATG AAG CCC CCA GCA GAA GTG GTG OCT GGG CAC CTC ATC ACA OGA CTA CTG GAC  
 MET Lys Pro Pro Ala Glu Val Val Pro Gly His Leu Ile Thr Arg Leu Leu Asp

1017                      1032                      1047  
 ACG AGA CTG GTC CAC CAC AAT GTG ACA OGG TGG GAA ACT TTT GAT GTG AGC OCT  
 Thr Arg Leu Val His His Asn Val Thr Arg Trp Glu Thr Phe Asp Val Ser Pro

1062                      1077                      1092                      1107  
 GCG GTC CTT OGC TGG ACC OGG GAG AAG CAG CCA AAC TAT GGG CTA GOC ATT GAG  
 Ala Val Leu Arg Trp Thr Arg Glu Lys Gln Pro Asn Tyr Gly Leu Ala Ile Glu

1122                      1137                      1152                      1167  
 GTG ACT CAC CTC CAT CAG ACT OGG ACC CAC CAG GGC CAG CAT GTC AGG ATT AGC  
 Val Thr His Leu His Gln Thr Arg Thr His Gln Gly Gln His Val Arg Ile Ser

1182                      1197                      1212  
 OGA TOG TTA OCT CAA GGG AGT GGG AAT TGG GOC CAG CTC OGG COC CTC CTG GTC  
 Arg Ser Leu Pro Gln Gly Ser Gly Asn Trp Ala Gln Leu Arg Pro Leu Leu Val

1227                      1242                      1257                      1272  
 ACC TTT GGC CAT GAT GGC OGG GGC CAT GOC TTG ACC OGA OGC OGG AGG GOC AAG  
 Thr Phe Gly His Asp Gly Arg Gly His Ala Leu Thr Arg Arg Arg Arg Ala Lys

1287                      1302                      1317  
 OGT AGC OCT AAG CAT CAC TCA CAG OGG GOC AGG AAG AAG AAT AAG AAC TGC OGG  
 Arg Ser Pro Lys His His Ser Gln Arg Ala Arg Lys Lys Asn Lys Asn Cys Arg

1332                      1347                      1362                      1377  
 OGC CAC TOG CTC TAT GTG GAC TTC AGC GAT GTG GGC TGG AAT GAC TGG ATT GTG  
 Arg His Ser Leu Tyr Val Asp Phe Ser Asp Val Gly Trp Asn Asp Trp Ile Val

1392                      1407                      1422                      1437  
 GCC CCA CCA GGC TAC CAG GOC TTC TAC TGC CAT GGG GAC TGC COC TTT OCA CTG  
 Ala Pro Pro Gly Tyr Gln Ala Phe Tyr Cys His Gly Asp Cys Pro Phe Pro Leu

1452 1467 1482  
GCT GAC CAC CTC AAC TCA ACC AAC CAT GCC ATT GTG CAG ACC CTG GTC AAT TCT  
Ala Asp His Leu Asn Ser Thr Asn His Ala Ile Val Gln Thr Leu Val Asn Ser

1497 1512 1527 1542  
GTC AAT TCC AGT ATC CCC AAA GOC TGT TGT GTG CCC ACT GAA CTG AGT GOC ATC  
Val Asn Ser Ser Ile Pro Lys Ala Cys Cys Val Pro Thr Glu Leu Ser Ala Ile

1557 1572 1587  
TCC ATG CTG TAC CTG GAT GAG TAT GAT AAG GTG GTA CTG AAA AAT TAT CAG GAG  
Ser MET Leu Tyr Leu Asp Glu Tyr Asp Lys Val Val Leu Lys Asn Tyr Gln Glu

1602 1617 (408) 1636 1646 1656  
ATG GTA GTA GAG GGA TGT GGG TGC GGC TGAGATCAGG CAGTCCITGA GGATAGACAG  
MET Val Val Glu Gly Cys Gly Cys Arg

1666 1676 1686 1696 1706 1716 1726  
ATATACACAC CACACACACA CACCACATAC ACCACACACA CAOGITCCCA TCCACTCACC CACACACTAC

1736 1746 1756 1766 1776 1786 1796  
ACAGACTGCT TCCTTATAGC TGGACTTTTA TTTAAAAAAA AAAAAAATAA AATGGAAAAA ATCCCTAAAC

1806 1816 1826 1836 1846 1856 1866  
ATTCAOCTTG ACCTTATTTA TGACTTTAOG TGCAAATGTT TTGACCATAT TGATCATATA TTTTGACAAA

1876 1886 1896 1906 1916 1926 1936  
ATATATTTAT AACTAOGTAT TAAAGAAAA AAATAAAATG AGTCATTATT TTAAAAAATA AAAAAAACT

1946  
CTAGAGTOGA CGGAATTC

The sequences of BMP-2 Class I and II, as well as BMP-3 as shown in Tables III, IV, VII and VIII have significant homology to the beta (B) and beta (A) subunits of the inhibins. The inhibins are a family of hormones which are presently being investigated for use in contraception. See, A. J. Mason et al, Nature, 318:659-663 (1985). To a lesser extent they are also homologous to Mullerian inhibiting substance (MIS), a testicular glycoprotein that causes regression of the Mullerian duct during development of the male embryo and transforming growth factor-beta (TGF-b) which can inhibit or stimulate growth of cells or cause them to differentiate. Furthermore, the sequence of Table VII encoding hBMP-2 Class II has significant homology to the Drosophila decapentaplegic (DPP-C) locus transcript. See, J. Massague, Cell, 49:437-438 (1987); R. W. Padgett et al, Nature, 325:81-84 (1987); R. L. Cate et al, Cell 45: 685-698 (1986). It is considered possible therefore that BMP-2 Class II is the human homolog of the protein made from this transcript from this developmental mutant locus.

### C. BMP-3

Because bovine and human bone growth factor genes are presumed to be significantly homologous, oligonucleotide probes which have been shown to hybridize to the bovine DNA sequence of Table IV.A and IV.B are used to screen a human genomic library. A human genomic library (Toole et al., supra) is screened using these probes, and presumptive positives are isolated and DNA sequence obtained as described above. Evidence that this recombinant encodes a portion of the human bone inductive factor molecule relies on the bovine/human protein and gene structure homologies.

Once a recombinant bacteriophage containing DNA encoding a portion of the human BMP-3 molecule is obtained the human coding sequence is used as a probe as described in Example V (A) to identify a human cell line or tissue which synthesizes BMP-3. mRNA is selected by oligo (dT) cellulose

chromatography and cDNA is synthesized and cloned in lambda gt10 by established techniques (Toole et al., supra).

Alternatively, the entire gene encoding this human bone inductive factor can be identified and obtained in additional recombinant clones if necessary. Additional recombinants containing further 3' or 5' regions of this human bone inductive factor gene can be obtained by identifying unique DNA sequences at the end(s) of the original clone and using these as probes to rescreen the human genomic library. The gene can then be reassembled in a single plasmid by standard molecular biology techniques and amplified in bacteria. The entire human BMP-3 factor gene can then be transferred to an appropriate expression vector. The expression vector containing the gene is then transfected into a mammalian cell, e.g. monkey COS cells, where the human gene is transcribed and the RNA correctly spliced. Media from the transfected cells are assayed for bone inductive factor activity as described herein as an indication that the gene is complete. mRNA is obtained from these cells and cDNA synthesized from this mRNA source and cloned. The procedures described above may similarly be employed to isolate other species' bone inductive factor of interest by utilizing the bovine bone inductive factor and/or human bone inductive factor as a probe source. Such other species' bone inductive factor may find similar utility in, inter alia, fracture repair.

#### EXAMPLE VI

##### Expression of Bone Inductive Factors.

In order to produce bovine, human or other mammalian bone inductive factors, the DNA encoding it is transferred into an appropriate expression vector and introduced into mammalian cells by conventional genetic engineering techniques.

One skilled in the art can construct mammalian expression vectors by employing the sequence of Tables II-



VIII or other modified sequences and known vectors, such as pCD [Okayama et al., Mol. Cell Biol., 2:161-170 (1982)] and pJL3; pJL4 [Gough et al., EMBO J., 4:645-653 (1985)]. The transformation of these vectors into appropriate host cells can result in expression of osteoinductive factors. One skilled in the art could manipulate the sequences of Tables II-VIII by eliminating or replacing the mammalian regulatory sequences flanking the coding sequence with bacterial sequences to create bacterial vectors for intracellular or extracellular expression by bacterial cells. For example, the coding sequences could be further manipulated (e.g. ligated to other known linkers or modified by deleting non-coding sequences there-from or altering nucleotides therein by other known techniques). The modified bone inductive factor coding sequence could then be inserted into a known bacterial vector using procedures such as described in T. Taniguchi et al., Proc. Natl Acad. Sci. USA, 77:5230-5233 (1980). This exemplary bacterial vector could then be transformed into bacterial host cells and bone inductive factor expressed thereby. For a strategy for producing extracellular expression of bone inductive factor in bacterial cells., see, e.g. European patent application EPA 177,343.

Similar manipulations can be performed for the construction of an insect vector [See, e.g. procedures described in published European patent application 155,476] for expression in insect cells. A yeast vector could also be constructed employing yeast regulatory sequences for intracellular or extracellular expression of the factors of the present invention by yeast cells. [See, e.g., procedures described in published PCT application WO86/00639 and European patent application EPA 123,289].

A method for producing high levels of an osteoinductive factor of the invention from mammalian cells involves the construction of cells containing multiple copies of the heterologous bone inductive factor gene. The heterologous gene

can be linked to an amplifiable marker, e.g. the dihydrofolate reductase (DHFR) gene for which cells containing increased gene copies can be selected for propagation in increasing concentrations of methotrexate (MTX) according to the procedures of Kaufman and Sharp, J. Mol. Biol., 159:601-629 (1982). This approach can be employed with a number of different cell types.

For example, a plasmid containing a DNA sequence for a bone inductive factor of the invention in operative association with other plasmid sequences enabling expression thereof and the DHFR expression plasmid pAdA26SV(A)3 [Kaufman and Sharp, Mol. Cell. Biol., 2:1304 (1982)] can be co-introduced into DHFR-deficient CHO cells, DUKX-BII, by calcium phosphate coprecipitation and transfection. DHFR expressing transformants are selected for growth in alpha media with dialyzed fetal calf serum, and subsequently selected for amplification by growth in increasing concentrations of MTX (sequential steps in 0.02, 0.2, 1.0 and 5uM MTX) as described in Kaufman et al., Mol Cell Biol., 5:1750 (1983). Transformants are cloned, and biologically active bone inductive factor expression is monitored by rat bone formation assay. Bone inductive factor expression should increase with increasing levels of MTX resistance. Similar procedures can be followed to produce other bone inductive factors.

Alternatively, the human gene is expressed directly, as described above. Active bone inductive factor may be produced in bacteria or yeast cells. However the presently preferred expression system for biologically active recombinant human bone inductive factor is stably transformed CHO cells.

As one specific example, to produce the human bone inductive factor (hBMP-1) of Example V, the insert of U2OS-1 is released from the vector arms by digestion with Sal I and subcloned into the mammalian expression vector pMT2CX digested with Xho I. Plasmid DNA from this subclone is transfected into COS cells by the DEAE-dextran procedure [Sompayrac and

Danna PNAS 78:7575-7578 (1981); Luthman and Magnusson, Nucl.Acids Res. 11: 1295-1308 (1983)]. Serum-free 24 hr. conditioned medium is collected from the cells starting 40-70 hr. post-transfection.

The mammalian expression vector pMT2 Cla-Xho (pMT2 CX) is a derivative of p91023 (b) (Wong et al., Science 228:810-815, 1985) differing from the latter in that it contains the ampicillin resistance gene in place of the tetracycline resistance gene and further contains a XhoI site for insertion of cDNA clones. The functional elements of pMT2 Cla-Xho have been described (Kaufman, R.J., 1985, Proc. Natl. Acad. Sci. USA 82:689-693) and include the adenovirus VA genes, the SV40 origin of replication including the 72 bp enhancer, the adenovirus major late promoter including a 5' splice site and the majority of the adenovirus tripartite leader sequence present on adenovirus late mRNAs, a 3' splice acceptor site, a DHFR insert, the SV40 early polyadenylation site (SV40), and pBR322 sequences needed for propagation in E. coli.

Plasmid pMT2 Cla-Xho is obtained by EcoRI digestion of pMT2-VWF, which has been deposited with the American Type Culture Collection (ATCC), Rockville, MD (USA) under accession number ATCC 67122. EcoRI digestion excises the cDNA insert present in pMT2-VWF, yielding pMT2 in linear form which can be ligated and used to transform E. coli HB 101 or DH-5 to ampicillin resistance. Plasmid pMT2 DNA can be prepared by conventional methods. pMT2CX is then constructed by digesting pMT2 with Eco RV and XbaI, treating the digested DNA with Klenow fragment of DNA polymerase I, and ligating Cla linkers (NEBiolabs, CATCGATG). This removes bases 2266 to 2421 starting from the Hind III site near the SV40 origin of replication and enhancer sequences of pMT2. Plasmid DNA is then digested with EcoRI, blunted as above, and ligated to an EcoRI adapter,

5' PO<sub>4</sub>-AATTCCTCGAGAGCT 3'

3' GGAGCTCTCGA 5'

digested with XhoI, and ligated, yielding pMT2 Cla-Xho, which may then be used to transform E. coli to ampicillin resistance. Plasmid pMT2 Cla-Xho DNA may be prepared by conventional methods.

#### Example VII

##### Biological Activity of Expressed Bone Inductive Factor

###### A. BMP-1

To measure the biological activity of the expressed bone inductive factor (hBMP-1) obtained in Example VI above. The factor is partially purified on a Heparin Sepharose column. 4 ml of transfection supernatant from one 100 mm dish is concentrated approximately 10 fold by ultrafiltration on a YM 10 membrane and then dialyzed against 20mM Tris, 0.15 M NaCl, pH 7.4 (starting buffer). This material is then applied to a 1.1 ml Heparin Sepharose column in starting buffer. Unbound proteins are removed by an 8 ml wash of starting buffer, and bound proteins, including BMP-1, are desorbed by a 3-4 ml wash of 20 mM Tris, 2.0 M NaCl, pH 7.4.

The proteins bound by the Heparin column are concentrated approximately 10-fold on a Centricon 10 and the salt reduced by diafiltration with 0.1% trifluoroacetic acid. The appropriate amount of this solution is mixed with 20 mg of rat matrix and then assayed for in vivo bone and cartilage formation as previously described in Example III. A mock transfection supernatant fractionation is used as a control.

The implants containing rat matrix to which specific amounts of human BMP-1 have been added are removed from rats after seven days and processed for histological evaluation. Representative sections from each implant are stained for the presence of new bone mineral with von Kossa and acid fuchsin, and for the presence of cartilage-specific matrix formation using toluidine blue. The types of cells present within the section, as well as the extent to which these cells display phenotype are evaluated.



Addition of human BMP-1 to the matrix material resulted in formation of cartilage-like nodules at 7 days post implantation. The chondroblast-type cells were recognizable by shape and expression of metachromatic matrix. The amount of activity observed for human BMP-1 was dependent upon the amount of human BMP-1 protein added to the matrix. Table IX illustrates the dose-response relationship of human BMP-1 protein to the amount of bone induction observed.

Table IX

<u>IMPLANT NUMBER</u>	<u>AMOUNT USED</u> (equivalent of ml transfection media)	<u>HISTOLOGICAL SCORE</u>
876-134-1	10 BMP-1	C+2
876-134-2	3 BMP-1	C+1
876-134-3	1 BMP-1	C +/-
876-134-4	10 MOCK	C -
876-134-5	3 MOCK	C -
876-134-6	1 MOCK	C -

Cartilage (c) activity was scored on a scale from 0(-) to 5.

Similar levels of activity are seen in the Heparin Sepharose fractionated COS cell extracts. Partial purification is accomplished in a similar manner as described above except that 6 M urea is included in all the buffers. Further, in a rat bone formation assay as described above, BMP-2 has similarly demonstrated chondrogenic activity.

The procedures described above may be employed to isolate other bone inductive factors of interest by utilizing the bovine bone inductive factors and/or human bone inductive factors as a probe source. Such other bone inductive factors may find similar utility in, inter alia, fracture repair.

The foregoing descriptions detail presently preferred embodiments of the present invention. Numerous modifications



and variations in practice thereof are expected to occur to those skilled in the art upon consideration of these descriptions. Those modifications and variations are believed to be encompassed within the claims appended hereto.

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International Application No: PCT/

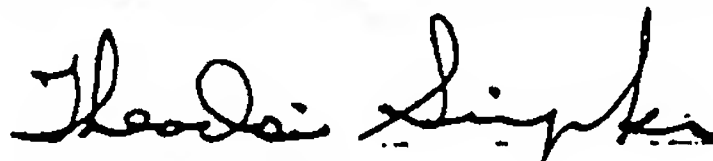
/

**MICROORGANISMS**Optional: Sheet in connection with the microorganism referred to on page \_\_\_\_\_, line \_\_\_\_\_ of the description <sup>1</sup>**A. IDENTIFICATION OF DEPOSIT <sup>2</sup>**Further deposits are identified on an additional sheet ☐ <sup>3</sup>Name of depositary institution <sup>4</sup>

American Type Culture Collection

Address of depositary institution (including postal code and country) <sup>4</sup>12301 Parklawn Drive  
Rockville, Maryland 20852 USA

<u>Name of Deposit</u>	<u>ATCC No.</u>	<u>Referred to on page/line</u>	<u>Date of Deposit</u>
LP-H1	40311	29/20	March 4, 1987
bP50	40295	20/3	December 15, 1986
bP-21	40310	22/18	March 4, 1987
U2OS-3	40342	44/22	June 16, 1987
Lambda U2-OS-1	40343	32/33	June 16, 1987
Lambda BP819	40344	25/23	June 16, 1987
U2OS-39	40345	39/21	June 16, 1987

**C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE <sup>5</sup>** (if the indications are not for all designated States)**D. SEPARATE FURNISHING OF INDICATIONS <sup>6</sup>** (leave blank if not applicable)The indications listed below will be submitted to the International Bureau later <sup>9</sup> (Specify the general nature of the indications e.g., "Accession Number of Deposit")E. ☒ This sheet was received with the international application when filed (to be checked by the receiving Office)  
(Authorized Officer)☐ The date of receipt (from the applicant) by the International Bureau <sup>10</sup>

(Authorized Officer)

## WHAT IS CLAIMED IS:

- 1.. A pharmaceutical composition comprising a protein selected from the group consisting of:
  - (a) BMP-1;
  - (b) BMP-2 Class I;
  - (c) BMP-2 Class II;
  - (d) BMP-3; andmixtures thereof, in a pharmaceutically acceptable vehicle.
- 2.. A composition of Claim 1 wherein said protein is BMP-1.
- 3.. A composition of Claim 1 wherein said protein is BMP-2 Class I.
4. A composition of Claim 1 wherein said protein is BMP-2 Class II.
5. A composition of Claim 1 wherein said protein is BMP-3.
6. The pharmaceutical composition of Claim 1 further comprising a matrix capable of delivering the composition to the site of the bone defect and providing a structure for inducing bone formation.
- 7.. The composition of Claim 6 wherein said matrix comprises a material selected from the group consisting of hydroxyapatite, collagen, polylactic acid and tricalcium phosphate.
- 8.. A method for inducing bone formation in a patient in need of same comprising administering to said patient an effective amount of a composition of Claim 1-7.
9. A process for producing BMP-1 comprising culturing in a suitable culture medium a cell line transformed with a DNA sequence encoding BMP-1, said DNA sequence being in relative association with an expression control sequence therefor, and isolating BMP-1 from said culture medium.
10. A process according to Claim 9 wherein said DNA sequence comprises substantially the nucleotide sequence of Table VI.
11. A process for producing BMP-2 Class I comprising culturing in a suitable culture medium a cell line transformed

with a DNA sequence encoding BMP-2 Class I, said DNA sequence being in relative association with an expression control sequence therefor, and isolating BMP-2 Class I from said culture medium.

12. A process for according to Claim 11 wherein said DNA sequence comprises substantially the nucleotide sequence of Table VII.

13.. A process for producing BMP-2 Class II comprising culturing in a suitable culture medium a cell line transformed with a DNA sequence encoding BMP-2 Class II, said DNA sequence being in relative association with an expression control sequence therefor, and isolating BMP-2 Class II from said culture medium.

14. A process according to Claim 13 wherein said DNA sequence comprises substantially the nucleotide sequence of Table VIII.

15. A process for producing BMP-3 comprising culturing in a suitable culture medium a cell line transformed with a DNA sequence encoding BMP-3, said DNA sequence being in relative association with an expression control sequence therefor and isolating BMP-3 from said culture medium.

16. A process according to Claim 15 wherein said DNA sequence comprises substantially the nucleotide sequence of Table IVA and IVB.

17. A cDNA sequence encoding BMP-1 comprising substantially the nucleotide sequence of Table VI or a sequence which hybridize thereto under stringent conditions and which upon expression codes for a protein exhibiting substantial properties of BMP-1.

18. A cDNA sequence encoding BMP-2 Class I comprising substantially the nucleotide sequence of Table VII or a sequence which hybridizes thereto under stringent conditions and which upon expression codes for a protein exhibiting substantial properties of BMP-2 Class I.

19. A cDNA sequence encoding BMP-2 Class II comprising

substantially the nucleotide sequence of Table VIII or a sequence which hybridizes thereto under stringent conditions and which upon expression codes for a protein exhibiting substantial properties of BMP-2 Class II.

20. A cDNA sequence encoding BMP-3 comprising substantially the nucleotide sequence of Table IVA and IVB or a sequence which hybridizes thereto under stringent conditions and which upon expression codes for a protein exhibiting substantial properties of BMP-3.

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## AMENDED CLAIMS

[received by the International Bureau on 8 December 1987 (08.12.87)  
original claims 6, 8, 10, 12, 14, 16-20 amended;  
new claims 21-23 added; other claims unchanged (13 pages)]

1. A pharmaceutical composition comprising a protein selected from the group consisting of:
  - (a) BMP-1;
  - (b) BMP-2 Class I;
  - (c) BMP-2 Class II;
  - (d) BMP-3; andmixtures thereof, in a pharmaceutically acceptable vehicle.
- 2.. A composition of Claim 1 wherein said protein is BMP-1..
- 3.. A composition of Claim 1 wherein said protein is BMP-2 Class I.
4. A composition of Claim 1 wherein said protein is BMP-2 Class II.
5. A composition of Claim 1 wherein said protein is BMP-3.
6. The pharmaceutical composition of Claim 1 further comprising a matrix capable of delivering the composition to the site of the bone or cartilage defect and providing a structure for inducing bone or cartilage formation.
7. The composition of Claim 6 wherein said matrix comprises a material selected from the group consisting of hydroxyapatite, collagen, polylactic acid and tricalcium phosphate.
8. A method for inducing bone or cartilage formation in a patient in need of same comprising administering to said patient an effective amount of a composition of Claim 1-7.

9. A process for producing BMP-1 comprising the steps of culturing in a suitable culture medium a host cell transformed with a DNA sequence encoding BMP-1, said DNA sequence being in relative association with an expression control sequence therefor; and isolating said BMP-1 therefrom.

10. A process according to Claim 9 wherein said DNA sequence comprises substantially the nucleotide sequence as follows:

10	20	30	50
CTAGAGGCG	CTTCCTCGC	CGCGGCGCG	CCAGC ATG CCC GGC GTG GCC OGC CTG CCG
			MET Pro Gly Val Ala Arg Leu Pro
65	80	95	110
CTG CTG CTC GGG CTG CTG CTG CTC CCG CGT CCC GGC OGC CCG CTG GAC TTG GCC			
Leu Leu Leu Gly Leu Leu Leu Leu Pro Arg Pro Gly Arg Pro Leu Asp Leu Ala			
125	140	155	
GAC TAC ACC TAT GAC CTG GCG GAG GAG GAC GAC TCG GAG CCC CTC AAC TAC AAA			
Asp Tyr Thr Tyr Asp Leu Ala Glu Glu Asp Asp Ser Glu Pro Leu Asn Tyr Lys			
170	185	200	215
GAC CCC TGC AAG GCG GCT GCC TTT CTT GGG GAC ATT GCC CTG GAC GAA GAG GAC			
Asp Pro Cys Lys Ala Ala Ala Phe Leu Gly Asp Ile Ala Leu Asp Glu Glu Asp			
230	245	260	275
CTG AGG GCC TTC CAG GTA CAG CAG GCT GTG GAT CTC AGA OGC CAC ACA GCT CGT			
Leu Arg Ala Phe Gln Val Gln Gln Ala Val Asp Leu Arg Arg His Thr Ala Arg			
290	305	320	
AAG TCC TCC ATC AAA GCT GCA GTT CCA GGA AAC ACT TCT ACC CCC AGC TGC CAG			
Lys Ser Ser Ile Lys Ala Ala Val Pro Gly Asn Thr Ser Thr Pro Ser Cys Gln			
335	350	365	380
AGC ACC AAC GGG CAG CCT CAG AGG GGA GCC TGT GGG AGA TCG AGA GGT AGA TCC			
Ser Thr Asn Gly Gln Pro Gln Arg Gly Ala Cys Gly Arg Trp Arg Gly Arg Ser			
395	410	425	
CGT AGC OGC OGC GCG GCG ACG TCC CGA CCA GAG CGT GTG TCG CCC GAT GGG GTC			
Arg Ser Arg Arg Ala Ala Thr Ser Arg Pro Glu Arg Val Trp Pro Asp Gly Val			
440	455	470	485
ATC CCC TTT GTC ATT GGG GGA AAC TTC ACT GGT AGC CAG AGG GCA GTC TTC OGC			
Ile Pro Phe Val Ile Gly Gly Asn Phe Thr Gly Ser Gln Arg Ala Val Phe Arg			
500	515	530	545
CAG GCC ATG AGG CAC TGG GAG AAG CAC ACC TGT GTC ACC TTC CTG GAG OGC ACT			
Gln Ala MET Arg His Trp Glu Lys His Thr Cys Val Thr Phe Leu Glu Arg Thr			

560 575 590  
 GAC GAG GAC AGC TAT ATT GTG TTC ACC TAT OGA CCT TGC GGG TGC TGC TOC TAC  
 Asp Glu Asp Ser Tyr Ile Val Phe Thr Tyr Arg Pro Cys Gly Cys Cys Ser Tyr

605 620 635 650  
 GTG GGT OGC OGC GGC GGG GGC CCC CAG GCC ATC TCC ATC GGC AAG AAC TGT GAC  
 Val Gly Arg Arg Gly Gly Gly Pro Gln Ala Ile Ser Ile Gly Lys Asn Cys Asp

665 680 695  
 AAG TTC GGC ATT GTG GTC CAC GAG CTG GGC CAC GTC GTC GGC TTC TGG CAC GAA  
 Lys Phe Gly Ile Val Val His Glu Leu Gly His Val Val Gly Phe Trp His Glu

710 725 740 755  
 CAC ACT OGG CCA GAC OGG GAC OGC CAC GTT TCC ATC GTT OGT GAG AAC ATC CAG  
 His Thr Arg Pro Asp Arg Asp Arg His Val Ser Ile Val Arg Glu Asn Ile Gln

770 785 800 815  
 CCA GGG CAG GAG TAT AAC TTC CTG AAG ATG GAG CCT CAG GAG GTG GAG TCC CTG  
 Pro Gly Gln Glu Tyr Asn Phe Leu Lys MET Glu Pro Gln Glu Val Glu Ser Leu

830 845 860  
 GGG GAG ACC TAT GAC TTC GAC AGC ATC ATG CAT TAC GCT OGG AAC ACA TTC TCC  
 Gly Glu Thr Tyr Asp Phe Asp Ser Ile MET His Tyr Ala Arg Asn Thr Phe Ser

875 890 905 920  
 AGG GGC ATC TTC CTG GAT ACC ATT GTC CCC AAG TAT GAG GTG AAC GGG GTG AAA  
 Arg Gly Ile Phe Leu Asp Thr Ile Val Pro Lys Tyr Glu Val Asn Gly Val Lys

935 950 965  
 CCT CCC ATT GGC CAA AGG ACA OGG CTC AGC AAG GGG GAC ATT GCC CAA GCC OGC  
 Pro Pro Ile Gly Gln Arg Thr Arg Leu Ser Lys Gly Asp Ile Ala Gln Ala Arg

980 995 1010 1025  
 AAG CTT TAC AAG TGC CCA GCC TGT GGA GAG ACC CTG CAA GAC AGC ACA GGC AAC  
 Lys Leu Tyr Lys Cys Pro Ala Cys Gly Glu Thr Leu Gln Asp Ser Thr Gly Asn

1040 1055 1070 1085  
 TTC TCC TCC CCT GAA TAC CCC AAT GGC TAC TCT GCT CAC ATG CAC TGC GTG TGG  
 Phe Ser Ser Pro Glu Tyr Pro Asn Gly Tyr Ser Ala His MET His Cys Val Trp

1100 1115 1130  
 OGC ATC TCT GTC ACA CCC GGG GAG AAG ATC ATC CTG AAC TTC ACG TCC CTG GAC  
 Arg Ile Ser Val Thr Pro Gly Glu Lys Ile Ile Leu Asn Phe Thr Ser Leu Asp

1145 1160 1175 1190  
 CTG TAC OGC AGC OGC CTG TGC TGG TAC GAC TAT GTG GAG GTC OGA GAT GGC TTC  
 Leu Tyr Arg Ser Arg Leu Cys Trp Tyr Asp Tyr Val Glu Val Arg Asp Gly Phe

1205 1220 1235  
 TGG AGG AAG GCG CCC CTC OGA GGC OGC TTC TGC GGG TCC AAA CTC CCT GAG CCT  
 Trp Arg Lys Ala Pro Leu Arg Gly Arg Phe Cys Gly Ser Lys Leu Pro Glu Pro

1250                      1265                      1280                      1295  
 ATC GTC TCC ACT GAC AGC CGC CTC TGG GTT GAA TTC CGC AGC AGC AGC AAT TGG  
 Ile Val Ser Thr Asp Ser Arg Leu Trp Val Glu Phe Arg Ser Ser Ser Asn Trp

1310                      1325                      1340                      1355  
 GTT GGA AAG GGC TTC TTT GCA GTC TAC GAA GCC ATC TGC GGG GGT GAT GTG AAA  
 Val Gly Lys Gly Phe Phe Ala Val Tyr Glu Ala Ile Cys Gly Gly Asp Val Lys

1370                      1385                      1400  
 AAG GAC TAT GGC CAC ATT CAA TCG CCC AAC TAC CCA GAC GAT TAC CGG CCC AGC  
 Lys Asp Tyr Gly His Ile Gln Ser Pro Asn Tyr Pro Asp Asp Tyr Arg Pro Ser

1415                      1430                      1445                      1460  
 AAA GTC TGC ATC TGG CGG ATC CAG GTG TCT GAG GGC TTC CAC GTG GGC CTC ACA  
 Lys Val Cys Ile Trp Arg Ile Gln Val Ser Glu Gly Phe His Val Gly Leu Thr

1475                      1490                      1505  
 TTC CAG TCC TTT GAG ATT GAG CGC CAC GAC AGC TGT GCC TAC GAC TAT CTG GAG  
 Phe Gln Ser Phe Glu Ile Glu Arg His Asp Ser Cys Ala Tyr Asp Tyr Leu Glu

1520                      1535                      1550                      1565  
 GTG CGC GAC GGG CAC AGT GAG AGC AGC ACC CTC ATC GGG CGC TAC TGT GGC TAT  
 Val Arg Asp Gly His Ser Glu Ser Ser Thr Leu Ile Gly Arg Tyr Cys Gly Tyr

1580                      1595                      1610                      1625  
 GAG AAG CCT GAT GAC ATC AAG AGC ACG TCC AGC CGC CTC TGG CTC AAG TTC GTC  
 Glu Lys Pro Asp Asp Ile Lys Ser Thr Ser Ser Arg Leu Trp Leu Lys Phe Val

1640                      1655                      1670  
 TCT GAC GGG TCC ATT AAC AAA GCG GGC TTT GCC GTC AAC TTT TTC AAA GAG GTG  
 Ser Asp Gly Ser Ile Asn Lys Ala Gly Phe Ala Val Asn Phe Phe Lys Glu Val

1685                      1700                      1715                      1730  
 GAC GAG TGC TCT CGC CCC AAC CGC GGG GGC TGT GAG CAG CGC TGC CTC AAC ACC  
 Asp Glu Cys Ser Arg Pro Asn Arg Gly Gly Cys Glu Gln Arg Cys Leu Asn Thr

1745                      1760                      1775  
 CTG GGC AGC TAC AAG TGC AGC TGT GAC CCC GGG TAC GAG CTG GCC CCA GAC AAG  
 Leu Gly Ser Tyr Lys Cys Ser Cys Asp Pro Gly Tyr Glu Leu Ala Pro Asp Lys

1790                      1805                      1820                      1835  
 CGC CGC TGT GAG GCT GCT TGT GGC GGA TTC CTC ACC AAG CTC AAC GGC TCC ATC  
 Arg Arg Cys Glu Ala Ala Cys Gly Gly Phe Leu Thr Lys Leu Asn Gly Ser Ile

1850                      1865                      1880                      1895  
 ACC AGC CGC GGC TGG CCC AAG GAG TAC CCC CCC AAC AAG AAC TGC ATC TGG CAG  
 Thr Ser Pro Gly Trp Pro Lys Glu Tyr Pro Pro Asn Lys Asn Cys Ile Trp Gln

1910                      1925                      1940  
 CTG GTG GGC CCC ACC CAG TAC CGC ATC TCC CTG CAG TTT GAC TTC TTT GAG ACA  
 Leu Val Ala Pro Thr Gln Tyr Arg Ile Ser Leu Gln Phe Asp Phe Phe Glu Thr

1955 1970 1985 2000  
GAG GGC AAT GAT GTG TGC AAG TAC GAC TTC GTG GAG GTG OGC AGT GGA CTC ACA  
Glu Gly Asn Asp Val Cys Lys Tyr Asp Phe Val Glu Val Arg Ser Gly Leu Thr

2015 2030 2045  
GCT GAC TCC AAG CTG CAT GGC AAG TTC TGT GGT TCT GAG AAG CCC GAG GTC ATC  
Ala Asp Ser Lys Leu His Gly Lys Phe Cys Gly Ser Glu Lys Pro Glu Val Ile

2060 2075 2090 2105  
ACC TCC CAG TAC AAC AAC ATG OGC GTG GAG TTC AAG TCC GAC AAC ACC GTG TCC  
Thr Ser Gln Tyr Asn Asn MET Arg Val Glu Phe Lys Ser Asp Asn Thr Val Ser

2120 2135 2150 2165  
AAA AAG GGC TTC AAG GCC CAC TTC TTC TCA GAA AAG AGG CCA GCT CTG CAG CCC  
Lys Lys Gly Phe Lys Ala His Phe Phe Ser Glu Lys Arg Pro Ala Leu Gln Pro

2180 2195 2210  
CCT OGC GGA OGC CCC CAC CAG CTC AAA TTC OGA GTG CAG AAA AGA AAC OGC ACC  
Pro Arg Gly Arg Pro His Gln Leu Lys Phe Arg Val Gln Lys Arg Asn Arg Thr

2225 2235 2245 2255 2265 2275 2285  
CCC CAG TGAGGCCTGC CAGGCCTCCC GGACCCCTTG TTAATCAGGA ACCTCACCTT GGACGGAATG  
Pro Gln

2295 2305 2315 2325 2335 2345 2355  
GGATGGGGGC TTOGGTGCCC ACCAACCCCC CACCTCCACT CTGCCATTCC GGCCACCTC CCTCTGGCOG

2365 2375 2385 2395 2405 2415 2425  
GACAGAACTG GTGCTCTCTT CTCCCCACTG TGCCCGTCCG OGGACOGGGG ACCCTTCCCC GTGCCCTACC

2435 2445 2455 2465 2475 2485 2495  
CCCTCCCATT TTGATGGTGT CTGTGACATT TCCTGTGTGT AAGTAAAAGA GGGACCCCTG CGTCCTGCCT

CTAGA

11. A process for producing BMP-2 Class I comprising culturing in a suitable culture medium a cell line transformed with a DNA sequence encoding BMP-2 Class I, said DNA sequence being in relative association with an expression control sequence therefor, and isolating BMP-2 Class I from said culture medium.

12. A process for according to Claim 11 wherein said DNA sequence comprises substantially the nucleotide sequence as



follows:

10	20	30	40	50	60	70
GTOGACTCTA	GAGTGTGTGT	CAGCACTTGG	CTGGGGACTT	CTTGAACCTG	CAGGGAGAAT	AACTTGCGCA
80	90	100	110	120	130	140
CCCCACTTTG	OGGOGGTGOC	TTTGCCCCAG	OGGAGCCTGC	TTOGCCATCT	COGAGCCCCA	COGCCCCCTCC
150	160	170	180	190	200	210
ACTCCTGGGC	CTTGCCCGAC	ACTGAGAOGC	TGTTCCCGAG	GTGAAAAGAG	AGACTGOGCG	GCOGGCACCC
220	230	240	250	260	270	280
GGGAGAAGGA	GGAGGCAAAG	AAAAGGAACG	GACATTGGGT	CCTTGOGCCA	GGTCCTTTGA	CCAGAGTTTT
290	300	310	320	330	340	350
TCCATGTGGA	CGCTCTTTCA	ATGGAGGTGT	CCCGOGGTGC	TTCTTAGAAG	GACTGOGGTC	TCCTAAAGGT
370						
OGAACC	ATG	GTG	GCC	GGG	ACC	OGC
MET Val Ala Gly Thr Arg Cys Leu Leu Ala Leu Leu Leu Pro Gln Val						
385						
400						
415						
CTC	CTG	GGC	GGC	GCG	GCT	GGC
Leu	Leu	Gly	Gly	Ala	Ala	Gly
430						
CTC	GTT	COG	GAG	CTG	GGC	OGC
Leu	Val	Pro	Glu	Leu	Gly	Arg
445						
AGG	AAG	TTC	GCG			
Arg	Lys	Phe	Ala			
460						
GCG	GCG	TOG	TOG	GGC	OGC	CCC
Ala	Ala	Ser	Ser	Gly	Arg	Pro
475						
TCA	TCC	CAG	CCC	TCT	GAC	GAG
Ser	Ser	Gln	Pro	Ser	Asp	Glu
490						
GAG	GTC	CTG	AGC	GAG		
Val	Leu	Ser	Glu			
505						
TTC	GAG	TTG	OGG	CTG	CTC	AGC
Phe	Glu	Leu	Arg	Leu	Leu	Ser
520						
AGC	ATG	TTC	GGC	CTG	AAA	CAG
Ser	MET	Phe	Gly	Leu	Lys	Gln
535						
AGA	CCC	AOC	CCC	AGC		
Arg	Pro	Thr	Pro	Ser		
550						
AGG	GAC	TOG	GGT			
Arg	His	Ser	Gly			
565						
AGG	GAC	GCC	GTG	GTG	CCC	CCC
Arg	Asp	Ala	Val	Val	Pro	Pro
580						
TAC	ATG	CTA	GAC	CTG	TAT	OGC
Tyr	MET	Leu	Asp	Leu	Tyr	Arg
595						
AGG	CAC	TOG	GGT			
Arg	His	Ser	Gly			
610						
CAG	COG	GGC	TCA	CCC	GCC	CCA
Gln	Pro	Gly	Ser	Pro	Ala	Pro
625						
GAC	CAC	OGG	TTG	GAG	AGG	GCA
His	Arg	Leu	Glu	Arg	Ala	Ala
640						
AGC	OGC	AGC	TTC	CAC	CAT	GAA
Ser	Arg	Phe	His	His	Glu	Glu
655						
TCT	TTG	GAA	GAA	CTA	CCA	GAA
Ser	Leu	Glu	Glu	Leu	Pro	Glu
670						
ACG						
Thr						
685						
AGT	GGG	AAA	ACA	ACC	OGG	AGA
Ser	Gly	Lys	Thr	Thr	Arg	Arg
700						
TTC	TTC	TTT	AAT	TTA	AGT	TCT
Phe	Phe	Phe	Asn	Leu	Ser	Ser
715						
ATC	CCC	ACG	GAG			
Ile	Pro	Thr	Glu			
730						
GAG	TTT	ATC	ACC	TCA	GCA	GAG
Glu	Phe	Ile	Thr	Ser	Ala	Glu
745						
CTT	CAG	GTT	TTC	OGA	GAA	CAG
Leu	Gln	Val	Phe	Arg	Glu	Gln
760						
ATG	CAA	GAT	GCT			
Met	Gln	Asp	Ala			
775						
GAG						
Glu						
790						
GAG	TTT	ATC	ACC	TCA	GCA	GAG
Glu	Phe	Ile	Thr	Ser	Ala	Glu
805						
CTT	CAG	GTT	TTC	OGA	GAA	CAG
Leu	Gln	Val	Phe	Arg	Glu	Gln
820						
ATG	CAA	GAT	GCT			
Met	Gln	Asp	Ala			
835						

										850				865				880							
TTA	GGA	AAC	AAT	AGC	AGT	TTC	CAT	CAC	CGA	ATT	AAT	ATT	TAT	GAA	ATC	ATA	AAA								
Leu	Gly	Asn	Asn	Ser	Ser	Phe	His	His	Arg	Ile	Asn	Ile	Tyr	Glu	Ile	Ile	Lys								
										895				910				925				940			
CCT	GCA	ACA	GCC	AAC	TCG	AAA	TTC	CCC	GTG	ACC	AGT	CTT	TTG	GAC	ACC	AGG	TTG								
Pro	Ala	Thr	Ala	Asn	Ser	Lys	Phe	Pro	Val	Thr	Ser	Leu	Leu	Asp	Thr	Arg	Leu								
										955				970				985							
GTG	AAT	CAG	AAT	GCA	AGC	AGG	TGG	GAA	AGT	TTT	GAT	GTC	ACC	CCC	GCT	GTG	ATG								
Val	Asn	Gln	Asn	Ala	Ser	Arg	Trp	Glu	Ser	Phe	Asp	Val	Thr	Pro	Ala	Val	MET								
										1000				1015				1030				1045			
CGG	TGG	ACT	GCA	CAG	GGA	CAC	GCC	AAC	CAT	GGA	TTC	GTG	GTG	GAA	GTG	GCC	CAC								
Arg	Trp	Thr	Ala	Gln	Gly	His	Ala	Asn	His	Gly	Phe	Val	Val	Glu	Val	Ala	His								
										1060				1075				1090				1105			
TTG	GAG	GAG	AAA	CAA	GGT	GTC	TCC	AAG	AGA	CAT	GTT	AGG	ATA	AGC	AGG	TCT	TTG								
Leu	Glu	Glu	Lys	Gln	Gly	Val	Ser	Lys	Arg	His	Val	Arg	Ile	Ser	Arg	Ser	Leu								
										1120				1135				1150							
CAC	CAA	GAT	GAA	CAC	AGC	TGG	TCA	CAG	ATA	AGG	CCA	TTG	CTA	GTA	ACT	TTT	GGC								
His	Gln	Asp	Glu	His	Ser	Trp	Ser	Gln	Ile	Arg	Pro	Leu	Leu	Val	Thr	Phe	Gly								
										1165				1180				1195				1210			
CAT	GAT	GGA	AAA	GGG	CAT	CCT	CTC	CAC	AAA	AGA	GAA	AAA	CGT	CAA	GCC	AAA	CAC								
His	Asp	Gly	Lys	Gly	His	Pro	Leu	His	Lys	Arg	Glu	Lys	Arg	Gln	Ala	Lys	His								
										1225				1240				1255							
AAA	CAG	CGG	AAA	CGC	CTT	AAG	TCC	AGC	TGT	AAG	AGA	CAC	CCT	TTG	TAC	GTG	GAC								
Lys	Gln	Arg	Lys	Arg	Leu	Lys	Ser	Ser	Cys	Lys	Arg	His	Pro	Leu	Tyr	Val	Asp								
										1270				1285				1300				1315			
TTC	AGT	GAC	GTG	GGG	TGG	AAT	GAC	TGG	ATT	GTG	GCT	CCC	COG	GGG	TAT	CAC	GCC								
Phe	Ser	Asp	Val	Gly	Trp	Asn	Asp	Trp	Ile	Val	Ala	Pro	Pro	Gly	Tyr	His	Ala								
										1330				1345				1360				1375			
TTT	TAC	TGC	CAC	GGA	GAA	TGC	CCT	TTT	CCT	CTG	GCT	GAT	CAT	CTG	AAC	TCC	ACT								
Phe	Tyr	Cys	His	Gly	Glu	Cys	Pro	Phe	Pro	Leu	Ala	Asp	His	Leu	Asn	Ser	Thr								
										1390				1405				1420							
AAT	CAT	GCC	ATT	GTT	CAG	ACG	TTG	GTC	AAC	TCT	GTT	AAC	TCT	AAG	ATT	CCT	AAG								
Asn	His	Ala	Ile	Val	Gln	Thr	Leu	Val	Asn	Ser	Val	Asn	Ser	Lys	Ile	Pro	Lys								
										1435				1450				1465				1480			
GCA	TGC	TGT	GTC	COG	ACA	GAA	CTC	AGT	GCT	ATC	TCG	ATG	CTG	TAC	CTT	GAC	GAG								
Ala	Cys	Cys	Val	Pro	Thr	Glu	Leu	Ser	Ala	Ile	Ser	MET	Leu	Tyr	Leu	Asp	Glu								

1495                      1510                      1525  
 AAT GAA AAG GTT GTA TTA AAG AAC TAT CAG GAC ATG GTT GTG GAG GGT TGT GGG  
 Asn Glu Lys Val Val Leu Lys Asn Tyr Gln Asp MET Val Val Glu Gly Cys Gly  
  
 1540                      1553                      1563                      1573                      1583                      1593                      1603  
 TGT CGC TAGTACAGCA AAATTAAATA CATAAATATA TATATATATA TATATTTTAG AAAAAAGAAA  
 Cys Arg  
  
 AAAA

13. A process for producing BMP-2 Class II comprising culturing in a suitable culture medium a cell line transformed with a DNA sequence encoding BMP-2 Class II, said DNA sequence being in relative association with an expression control sequence therefor, and isolating BMP-2 Class II from said culture medium.

14. A process according to Claim 13 wherein said DNA sequence comprises substantially the nucleotide sequence as follows:

10	20	30	40	50	60	70
CTCTAGAGGG	CAGAGGAGGA	GGGAGGGAGG	GAAGGAGGCG	GGAGCCCGGC	COGGAAGCTA	GGTGAGTGTG
80	90	100	110	120	130	140
GCATCOGAGC	TGAGGGAOGC	GAGCCTGAGA	OGCOGCTGCT	GCTCOGGCTG	AGTATCTAGC	TTGTCTCCCC
150	160	170	180	190	200	210
GATGGGATTG	COGTCCAAGC	TATCTOGAGC	CTGCAGOGCC	ACAGTCCCCG	GCCCTOGCCC	AGGTTCACTG
220	230	240	250	260	270	280
CAACOGTTCA	GAGGTCCCCA	GGAGCTGCTG	CTGGOGAGCC	OGCTACTGCA	GGGACCTATG	GAGCCATTCC
290	300	310	320	330	340	350
GTAGTGCCAT	CCOGAGCAAC	GCACTGCTGC	AGCTTCCCTG	AGCCTTTCCA	GCAAGTTTGT	TCAAGATTGG
360	370	380	390	400		
CTGTCAAGAA	TCATGGACTG	TTATTATATG	CCTTGTTTTC	TGTCAAGACA	CC ATG ATT CCT	
					MET Ile Pro	
417	432	447	462			
GGT AAC CGA ATG CTG ATG GTC GTT TTA TTA TGC CAA GTC CTG CTA GGA GGC GCG						
Gly Asn Arg MET Leu MET Val Val Leu Leu Cys Gln Val Leu Leu Gly Gly Ala						
477	492	507				
AGC CAT GCT AGT TTG ATA CCT GAG ACG GGG AAG AAA AAA GTC GCC GAG ATT CAG						
Ser His Ala Ser Leu Ile Pro Glu Thr Gly Lys Lys Lys Val Ala Glu Ile Gln						

522                      537                      552                      567  
 GGC CAC GOG GGA GGA OGC OGC TCA GGG CAG AGC CAT GAG CTC CTG OGG GAC TTC  
 Gly His Ala Gly Gly Arg Arg Ser Gly Gln Ser His Glu Leu Leu Arg Asp Phe

                    582                      597                      612                      627  
 GAG GOG ACA CTT CTG CAG ATG TTT GGG CTG OGC OGC OGC COG CAG CCT AGC AAG  
 Glu Ala Thr Leu Leu Gln MET Phe Gly Leu Arg Arg Arg Pro Gln Pro Ser Lys

                    642                      657                      672  
 AGT GCC GTC ATT COG GAC TAC ATG OGG GAT CTT TAC OGG CTT CAG TCT GGG GAG  
 Ser Ala Val Ile Pro Asp Tyr MET Arg Asp Leu Tyr Arg Leu Gln Ser Gly Glu

                    687                      702                      717                      732  
 GAG GAG GAA GAG CAG ATC CAC AGC ACT GGT CTT GAG TAT CCT GAG OGC COG GCC  
 Glu Glu Glu Glu Gln Ile His Ser Thr Gly Leu Glu Tyr Pro Glu Arg Pro Ala

                    747                      762                      777  
 AGC OGG GCC AAC ACC GTG AGG AGC TTC CAC CAC GAA GAA CAT CTG GAG AAC ATC  
 Ser Arg Ala Asn Thr Val Arg Ser Phe His His Glu Glu His Leu Glu Asn Ile

792                      807                      822                      837  
 CCA GGG ACC AGT GAA AAC TCT GCT TTT OGT TTC CTC TTT AAC CTC AGC AGC ATC  
 Pro Gly Thr Ser Glu Asn Ser Ala Phe Arg Phe Leu Phe Asn Leu Ser Ser Ile

                    852                      867                      882                      897  
 CCT GAG AAC GAG GOG ATC TCC TCT GCA GAG CTT OGG CTC TTC OGG GAG CAG GTG  
 Pro Glu Asn Glu Ala Ile Ser Ser Ala Glu Leu Arg Leu Phe Arg Glu Gln Val

                    912                      927                      942  
 GAC CAG GGC CCT GAT TGG GAA AGG GGC TTC CAC OGT ATA AAC ATT TAT GAG GTT  
 Asp Gln Gly Pro Asp Trp Glu Arg Gly Phe His Arg Ile Asn Ile Tyr Glu Val

                    957                      972                      987                      1002  
 ATG AAG CCC CCA GCA GAA GTG GTG CCT GGG CAC CTC ATC ACA OGA CTA CTG GAC  
 MET Lys Pro Pro Ala Glu Val Val Pro Gly His Leu Ile Thr Arg Leu Leu Asp

                    1017                      1032                      1047  
 ACG AGA CTG GTC CAC CAC AAT GTG ACA OGG TGG GAA ACT TTT GAT GTG AGC CCT  
 Thr Arg Leu Val His His Asn Val Thr Arg Trp Glu Thr Phe Asp Val Ser Pro

1062                      1077                      1092                      1107  
 GOG GTC CTT OGC TGG ACC OGG GAG AAG CAG CCA AAC TAT GGG CTA GCC ATT GAG  
 Ala Val Leu Arg Trp Thr Arg Glu Lys Gln Pro Asn Tyr Gly Leu Ala Ile Glu

                    1122                      1137                      1152                      1167  
 GTG ACT CAC CTC CAT CAG ACT OGG ACC CAC CAG GGC CAG CAT GTC AGG ATT AGC  
 Val Thr His Leu His Gln Thr Arg Thr His Gln Gly Gln His Val Arg Ile Ser

                    1182                      1197                      1212  
 OGA TOG TTA OCT CAA GGG AGT GGG AAT TGG GCC CAG CTC OGG CCC CTC CTG GTC  
 Arg Ser Leu Pro Gln Gly Ser Gly Asn Trp Ala Gln Leu Arg Pro Leu Leu Val

1227                      1242                      1257                      1272  
 ACC TTT GGC CAT GAT GGC OGG GGC CAT GCC TTG ACC CGA CGC OGG AGG GCC AAG  
 Thr Phe Gly His Asp Gly Arg Gly His Ala Leu Thr Arg Arg Arg Arg Ala Lys

1287                      1302                      1317  
 CGT AGC OCT AAG CAT CAC TCA CAG OGG GCC AGG AAG AAG AAT AAG AAC TGC OGG  
 Arg Ser Pro Lys His His Ser Gln Arg Ala Arg Lys Lys Asn Lys Asn Cys Arg

1332                      1347                      1362                      1377  
 OGC CAC TCG CTC TAT GTG GAC TTC AGC GAT GTG GGC TGG AAT GAC TGG ATT GTG  
 Arg His Ser Leu Tyr Val Asp Phe Ser Asp Val Gly Trp Asn Asp Trp Ile Val

1392                      1407                      1422                      1437  
 GCC CCA CCA GGC TAC CAG GCC TTC TAC TGC CAT GGG GAC TGC CCC TTT CCA CTG  
 Ala Pro Pro Gly Tyr Gln Ala Phe Tyr Cys His Gly Asp Cys Pro Phe Pro Leu

1452                      1467                      1482  
 GCT GAC CAC CTC AAC TCA ACC AAC CAT GCC ATT GTG CAG ACC CTG GTC AAT TCT  
 Ala Asp His Leu Asn Ser Thr Asn His Ala Ile Val Gln Thr Leu Val Asn Ser

1497                      1512                      1527                      1542  
 GTC AAT TCC AGT ATC CCC AAA GCC TGT TGT GTG CCC ACT GAA CTG AGT GCC ATC  
 Val Asn Ser Ser Ile Pro Lys Ala Cys Cys Val Pro Thr Glu Leu Ser Ala Ile

1557                      1572                      1587  
 TCC ATG CTG TAC CTG GAT GAG TAT GAT AAG GTG GTA CTG AAA AAT TAT CAG GAG  
 Ser MET Leu Tyr Leu Asp Glu Tyr Asp Lys Val Val Leu Lys Asn Tyr Gln Glu

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1602                      1617                      1636                      1646                      1656                      1666  
 ATG GTA GTA GAG GGA TGT GGG TGC OGC TGAGATCAGG CAGTCCTTGA GGATAGACAG ATATACACAC  
 MET Val Val Glu Gly Cys Gly Cys Arg

1676                      1686                      1696                      1706                      1716                      1726                      1736  
 CACACACACA CACCACATAC ACCACACACA CAOGTTCCCA TCCACTCACC CACACACTAC ACAGACTGCT

1746                      1756                      1766                      1776                      1786                      1796                      1806  
 TCCTTATAGC TGGACTTTTA TTTAAAAAAA AAAAAAAAAA AATGGAAAAA ATCCCTAAAC ATTCACCTTG

1816                      1826                      1836                      1846                      1856                      1866                      1876  
 ACCTTATTTA TGACTTTAAG TCCAAATGTT TTGACCATAT TGATCATATA TTTTGACAAA ATATATTTAT

1886                      1896                      1906                      1916                      1926                      1936                      1946  
 AACTACGTAT TAAAGAAAA AAATAAAATG AGTCATTATT TTAAAAAAA AAAAAAACT CTAGAGTCGA  
 CGGAATTC

15. A process for producing BMP-3 comprising culturing in a suitable culture medium a cell line transformed with a DNA sequence encoding BMP-3, said DNA sequence being in relative



association with an expression control sequence therefor and isolating BMP-3 from said culture medium.

16. A process according to Claim 15 wherein said DNA sequence comprises substantially the nucleotide sequence as follows:

383	393	403	413	428
GAGGAGGAAG	CGGTCTAOGG	GGGTCTTCT	GCCTCTGCAG	AAC AAT GAG CTT CCT GGG GCA
				Asn Asn Glu Leu Pro Gly Ala
443	458	473	488	
GAA TAT CAG TAC AAG GAG GAT GAA GTA TGG GAG GAG AGG AAG CCT TAC AAG ACT				
Glu Tyr Gln Tyr Lys Glu Asp Glu Val Trp Glu Glu Arg Lys Pro Tyr Lys Thr				
503	518	533		
CTT CAG ACT CAG CCC CCT GAT AAG AGT AAG AAC AAA AAG AAA CAG AGG AAG GGA				
Leu Gln Thr Gln Pro Pro Asp Lys Ser Lys Asn Lys Lys Lys Gln Arg Lys Gly				
548	563	578	593	
CCT CAG CAG AAG AGT CAG ACG CTC CAG TTT GAT GAA CAG ACC CTG AAG AAG GCA				
Pro Gln Gln Lys Ser Gln Thr Leu Gln Phe Asp Glu Gln Thr Leu Lys Lys Ala				
608	623	638		
AGA AGA AAG CAA TGG ATT GAA CCC CGG AAT TGT GCC AGA CGG TAC CTT AAA GTG				
Arg Arg Lys Gln Trp Ile Glu Pro Arg Asn Cys Ala Arg Arg Tyr Leu Lys Val				
653	668	683	698	
GAC TTC GCA GAT ATT GGC TGG AGC GAA TGG ATT ATT TCC CCC AAG TCC TTC GAT				
Asp Phe Ala Asp Ile Gly Trp Ser Glu Trp Ile Ile Ser Pro Lys Ser Phe Asp				
713	728	743	756	766
GCC TAT TAC TGC TCC GGA GCG TGC CAG TTC CCC ATG CCA AAG GTAGCCATTG TTTTTGTCC				
Ala Tyr Tyr Cys Ser Gly Ala Cys Gln Phe Pro MET Pro Lys				
776	786			
TGTCCTTCCC	ATTTCATAG			
; and				

284	294	304	319
CTAACCTGTG	TTCTCCCTTT	TOGTTCTAG	TCT TTG AAG CCA TCA AAT CAC GCT ACC
			Ser Leu Lys Pro Ser Asn His Ala Thr

334	349	364	379
ATC CAG AGT ATA GTG AGA GCT GTG GGG GTC GTC CCT GGA ATC CCC GAG CCT TGC			
Ile Gln Ser Ile Val Arg Ala Val Gly Val Val Pro Gly Ile Pro Glu Pro Cys			

394	409	424	439
TGT GTG CCA GAA AAG ATG TCC TCA CTC AGC ATC TTA TTC TTT GAT GAA AAC AAG			
Cys Val Pro Glu Lys MET Ser Ser Leu Ser Ile Leu Phe Phe Asp Glu Asn Lys			

	454		469		484
AAT GIG GTA CTT AAA GTA TAT OCA AAC ATG ACA GTA GAG TCT TGT GCT TGC AGA					
Asn Val Val Leu Lys Val Tyr Pro Asn MET Thr Val Glu Ser Cys Ala Cys Arg					

503	513	523	533
TAACCTGGTG	AAGAACTCAT	CTGGATGCTT	AACTCAATCG.

17. A cDNA sequence encoding BMP-1 comprising substantially the nucleotide sequence recited in Claim 10 or a sequence which hybridizes thereto under stringent conditions and which upon expression codes for a protein exhibiting substantial properties of BMP-1.

18. A cDNA sequence encoding BMP-2 Class I comprising substantially the nucleotide sequence recited in Claim 12 or a sequence which hybridizes thereto under stringent conditions and which upon expression codes for a protein exhibiting substantial properties of BMP-2 Class I.

19. A cDNA sequence encoding BMP-2 Class II comprising substantially the nucleotide sequence recited in Claim 14 or a sequence which hybridizes thereto under stringent conditions and which upon expression codes for a protein exhibiting substantial properties of BMP-2 Class II.

20. A cDNA sequence encoding BMP-3 comprising substantially the nucleotide sequence recited in Claim 16 or a sequence which hybridizes thereto under stringent conditions and which upon expression codes for a protein exhibiting substantial properties of BMP-3.

21. A vector containing a DNA sequence encoding an osteoinductive protein and heterologous DNA, the DNA sequence encoding the protein being selected from the group consisting of:

a. a DNA sequence encoding BMP-1 comprising substantially the nucleotide sequence recited in Claim 10 or a sequence which

hybridize thereto under stringent conditions and which upon expression codes for a protein exhibiting substantial properties of BMP-1;

b. a DNA sequence encoding BMP-2 Class I comprising substantially the nucleotide sequence recited in Claim 12 or a sequence which hybridizes thereto under stringent conditions and which upon expression codes for a protein exhibiting substantial properties of BMP-2 Class I;

c. a DNA sequence encoding BMP-2 Class II comprising substantially the nucleotide sequence recited in Claim 14 or a sequence which hybridizes thereto under stringent conditions and which upon expression codes for a protein exhibiting substantial properties of BMP-2 Class II; and

d. a DNA sequence encoding BMP-3 comprising substantially the nucleotide sequence recited in Claim 16 or a sequence which hybridizes thereto under stringent conditions and which upon expression codes for a protein exhibiting substantial properties of BMP-3.

~~22.~~ A cell transformed with a vector according to claim 21 which is capable of expressing a DNA sequence encoding the osteoinductive protein and progeny of said cell.

23. The transformed cell according to claim 24 selected from the group consisting of a mammalian cell, a bacterial cell, an insect cell, and a yeast cell.

# INTERNATIONAL SEARCH REPORT

International Application No PCT/US87/01537

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) <sup>3</sup>

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): C07K 13/00, 15/00; A61K 37/00; See Attachment

US CL: 530/350, 395, 397; 514/12; 536/27 See Attachment

## II. FIELDS SEARCHED

Minimum Documentation Searched <sup>4</sup>

Classification System |

Classification Symbols

US	530/350, 395, 397; 514/12; 536/27 435/68, 70, 172.3; 935/13
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Documentation Searched other than Minimum Documentation  
to the Extent that such Documents are Included in the Fields Searched <sup>5</sup>

COMPUTER SEARCH CAS, APS: BONE MORPHOGEN, BONE  
INDUCTIVE PROTEIN, BMP, OSTEOINDUCTIVE FACTOR

## III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>14</sup>

Category <sup>6</sup>	Citation of Document, <sup>16</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No. <sup>14</sup>
<u>X, P</u> <u>Y, P</u>	US, A, 4,619,989 (URIST) 28 Oct 1986.	<u>1-8</u> <u>9-20</u>
<u>X</u> <u>Y</u>	US, A, 4,563,350 (NATHAN ET AL) 7 January 1986.	<u>1-8</u> <u>9-20</u>
<u>X</u> <u>Y</u>	US, A, 4,455,256 (URIST) 19 June 1984.	<u>1-8</u> <u>9-20</u>
X <u>    </u>	Proc. Natl. Acad. Sci USA, Vol. 81, issued January 1984, (Washington, D.C.),	1 <u>    </u>
Y	(URIST), "Purification of bovine morphogenetic protein by hydroxyapatite chromatography", pages 371-375.	2-20

\* Special categories of cited documents: <sup>13</sup>

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search <sup>2</sup>

08 October 1987

Date of Mailing of this International Search Report <sup>2</sup>

20 OCT 1987

International Searching Authority <sup>1</sup>

ISA/US

Signature of Authorized Officer <sup>20</sup>

*Alvin E. Tanenholtz*  
Alvin E. Tanenholtz

PCT/US87/01537

Attachment To Form PCT/ISA/210, Part I.

IPC(4): C12P 21/00, 21/02; C12N 15/00; C07H 15/12

US CL : 435/68, 70, 172.3; 935/13



## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, <sup>16</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No <sup>18</sup>
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Y	<u>Science</u> , Vol. 220 issued 13 May 1983 (Washington, D.C.) (URIST) "Bone cell Differentiation and Growth Factors" pages 680-686.	1-20
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Y	<u>Proc. Natl. Acad. Sci, USA</u> , Vol. 80 issued November 1983 (Washington, D.C.) (SAMPATH ET AL), "Homology of bone- inductive proteins from human monkey, bovine and rat extracellular matrix," pages 6591-6595.	1-20
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Y	<u>Proc. Natl. Acad. Sci, USA</u> , Vol. 78 issued November 1981, (Washington, D.C.) (SUGGS ET AL), "Use of synthetic oligonucleotides as hybridization probes: Isolation of cloned cDNA sequence for human $\beta_2$ -microglobulin" pages 6613-6617.	1-20
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